INFLUENCE OF AGE AND STRAIN ON REPRODUCTIVE PERFORMANCE OF THE BROILER BREEDER FEMALE

Nada Alzenbarakji

B.Sc. (Animal Production) Damascus University, Syria
Dipl. (Animal Production) Damascus University, Syria
M.Sc. (Poultry Husbandry) Damascus University, Syria

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Abstract

Chicken meat is an important source of high quality protein in the diet of most people in the world. Consequently, the increasing demand for this meat has made chicken meat production the most important growth sector among other meat species. This has been achieved by a half century of intensive genetic selection for growth traits; however, this was coupled with poor reproductive performance of broiler breeders.

Ross 708 represents a broiler breeder strain that has been developed for breast-meat yield, and has been reported to exhibit poor reproductive performance in comparison to Ross 308, a typical broiler breeder strain. Accordingly, the current study investigated key points involved in the reproductive process that might influence variation in reproductive performance. Ovarian follicles number was the first point to investigate, as they are the main material of the egg. Liver fatty acid profiles were also investigated in order to identify lipid metabolism and the efficiency of dietary fat utilisation, as the liver is the main site that supplies different body tissues with fatty acids. Carcass fat content was also examined as its negative relationship with reproduction is well documented. Finally, the content of calcium in the tibia bone was examined to identify whether variation in egg production was associated with differences in the metabolism of this element. Accordingly, broiler breeder females from Ross 308 and 708 strains, reared under the standard production system on two different commercial farms of PD Hook, were collected throughout the reproductive

cycle; starting at 25-week-old and in five weeks interval until 55-weeks of age.

Findings showed no difference in the number of both the large yellow follicles (P=0.332), and the small yellow follicles (P=0.134); whereas the number of large white follicles was higher in the 708 ovaries (P=0.005).

Differences in lipid metabolism were identified with a strong tendency for the 708s towards having lower content of linoleic acid (P=0.056) in addition to significantly lower α-linolenic acid (P=0.005). Of particular importance is the latter fatty acid as it is the precursor to (n-3) fatty acids, some of which were found to be less (P<0.001) in the 708s including docosapentaenoic (DPA) and docosahexaenoic (DHA). The importance of these fatty acids in follicular maturation is well documented in addition to the importance of the linoleic acid; these findings indicate that the 708s were not receiving adequate levels of the essential fatty acids which might have contributed to their poor reproductive performance. The 708s also laid down significantly more fat (P<0.001) in comparison to the 308s which could be another factor that has impaired their reproduction performance. This could indicate different levels of metabolic hormones which, in turn, have been found to act in concert with the reproductive hormones. 708s also exhibited a trend towards lower content of calcium in their tibiae, with an age by strain interaction and thus suggesting a difference in the metabolism of this element at some ages.

The current study has addressed changes of the investigated parameters with age, but the effect of genetic selection on reproductive performance was difficult to address. Rather, some physiological differences

have been identified; 708s were found to be receiving inadequate amounts of essential fatty acids, calcium content was found to be less at some ages and they exhibited a higher content of carcass fat. All these factors have the potential to contribute to poor reproductive performance, and once they are taken into consideration better assessment for the effect of the continued genetic selection for more growth traits can investigated.

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1.1 Introduction

In the early decades of the 20th century, raising chickens was an informal farmyard activity, with birds kept as a backyard flock for eggs, meat as an offshoot to egg production (Siegle & Wolford, 2003) and by-products such as feathers and manure. Thereafter, raising chickens for both eggs and meat gradually changed from backyard operations to intensive production in the second half of the 20th century where birds were kept in thousands (Figure1.1), with more efficient strains of birds being used to produce table eggs, and chicken meat strains that laid eggs needed to produce the F1 generation of meat chickens.

Figure 1.1 Raising chickens in the first half of the 20th century (left) and now (right) (http://www.chicken.org.au/page.php?id=5; 21.03.2010)

As a cheap source of high quality protein, poultry meat consumption rose, and continues to rise, steadily. In order to meet that increasing demand, the poultry industry has undertaken intensive genetic selection over the last few decades in order to breed chickens that grow faster than before. A typical broiler now reaches 1.8 kg body weight in 32 days with a feed conversion ratio of 1.47, whereas in the 1950s, a meat-type strain needed 101 days to reach this weight with a feed conversion of over 4.4 (Havenstein *et al.*, 2003a; Figure 1.2).

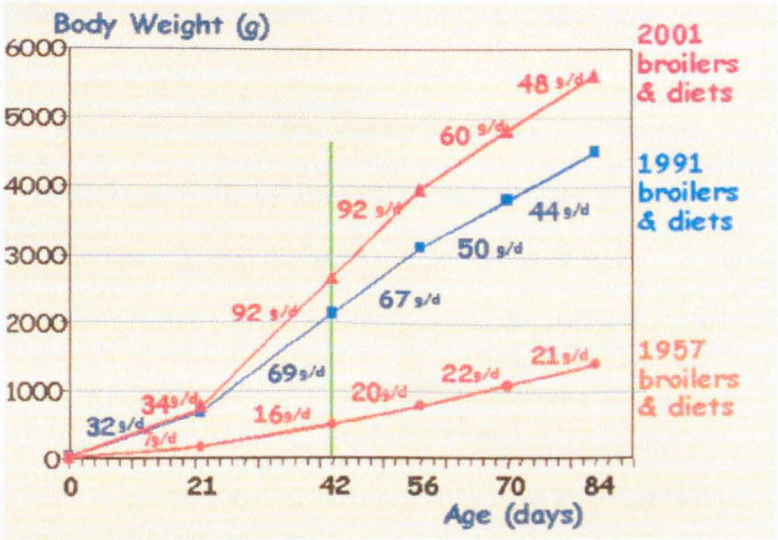


Figure 1.2 Change in growth rate (g/day) of broiler chickens from 1957-2001 (Adapted from Havenstein *et al.*, 1994; Havenstein *et al.*, 2003a)

Broiler chickens have been selected to grow faster and, more recently, to have greater yield of breast meat in order to meet consumer demand, for example the new Ross 708 strain. However, genetic change of such magnitude has inevitably had an impact on all aspects of the bird's physiological wellbeing. Female reproductive performance is an important aspect affected by genetic selection (Huey *et al.*, 1982; Burkhardt *et al.*, 1983).

1.2 Commercial broiler breeder flocks

It is important to clarify, initially, how supermarket chickens are produced, as these birds' parents are the subject of the current study. Males of two genetic pure lines developed for superior characteristics, such as good quality semen, fast feathering and disease resistance, are crossed with two pure female lines, developed for egg production, hatchability, and body conformation (Scrivener, 2009). Fertilized eggs resulting from this cross-breeding will give the great grandparent flocks (Pollock, 1999). The entire process is illustrated in Figure 1.3.

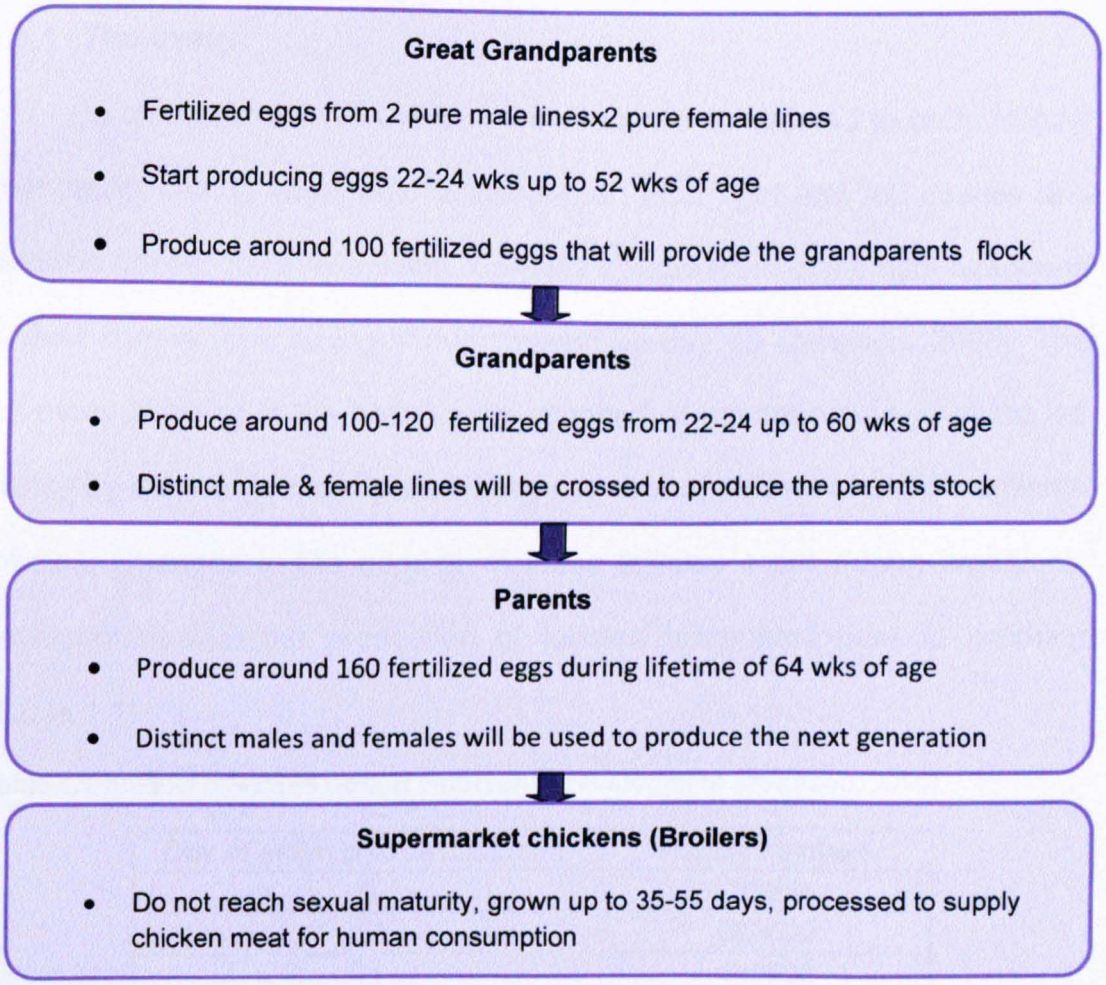


Figure 1.3 Entire process of commercial broiler breeder production (Etches, 1996)

1.3 Reproduction in broiler breeder females

The brain (hypothalamus; the master-controlling gland and anterior pituitary; adenohypophysis), liver (yolk formation), skeletal system (labile source of minerals needed for egg production), ovary (hierarchy of follicles) and oviduct (deposition of albumen, shell membranes and shell) all interact in the production of eggs. The role of each part of this system in egg production and the interaction of the different components in the regulation of the reproductive cycle in the hen will be discussed.

1.3.1 The ovary

In chickens, the left ovary and oviduct are considered to be functional post-hatch. During embryonic development, both right and left ovaries and oviducts are presumably evident, however a regression in the right ovary and oviduct commences during mid-incubation by day 10 (Johnson, 2000). The left ovary is found in the body cavity attached at the cephalic end of the left kidney by the mesovarian ligament and consists of a mass of undifferentiated follicles. Changes in the number of these follicles occur during embryonic development with the production of follicles terminated prior to hatching (Table 1.1).

Table 1.1 Follicle numbers during embryonic development (Johnson, 2000)

Day of embryonic development	Follicle numbers
9	28000
17	680000
21	480000

The ovary in the growing pullet is usually small and undeveloped and, therefore, quite difficult to find during necropsy (Leeson & Summers, 2000).

Once the bird reaches sexual maturity between 18-20 weeks old, the ovary looks like a cluster of grapes with follicles arranged in a clear hierarchy in which follicles can be classified into four categories according to size and colour (Johnson, 1990; Table 1.2, Figure 1.4).

Table 1.2 Ovarian follicle classification

Classification	Large yellow Follicles (LYF)		Small Yellow Follicles (SYF)		Large White Follicles LWF)		Small White Follicles (SWF)		Reference
Diameter/mm	Min	Max	Min	Max	Min	Max	Min	Max	
	10	-	5	10	2-5	5	-	1	Robinson *et al.*, 2003
	-	-	5	10	2	4	<1		Etches, 1993
	12	40	6	8	-	-	1	5	Johnson, 1990

Min=minimum, Max=maximum, (-) refers to unspecified diameter.

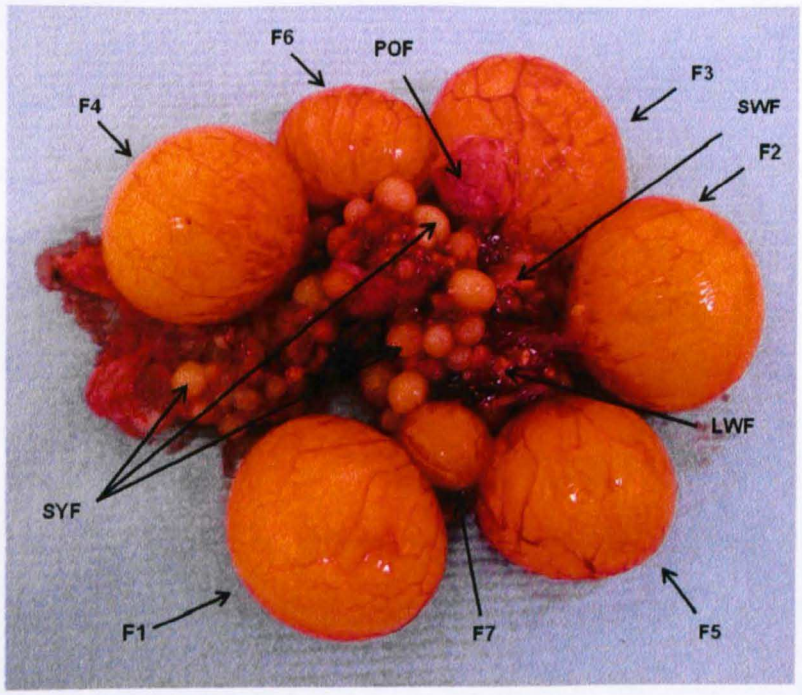


Figure 1.4 Left ovary of the hen, illustrating the hierarchal structure of ovarian follicles. F1, F2, F3, F4, F5, F6, F7 seven largest hierarchal follicles; POF, postovulatory follicle; LWF, large white follicles; SYF, small yellow follicles; SWF, small white follicles (definitions based on Johnson, 1990).

1.3.2 Sexual maturation and the onset of lay

Sexual maturity is a concert of precise changes that culminate in egg production. Dependent on the environment, endocrine changes occur leading to a major development in two aspects; the first is the secondary sexual characteristics, such as the comb develops and becomes reddened, prenuptial feathers grow, the distance between the pubic bones widen (Etches, 1996); the second is the development of the internal organs including the liver and its readiness to produce specific classes of lipid as yolk precursors (Deeley *et al.*, 1975), medullary bones, growth of the oviduct (Bogaard *et al.*, 1976). Around three weeks after the initiation of these signs, the first egg will be laid (Hudelson & Hudelson, 1996).

1.3.2.1 Age and body weight

Sexual maturity is controlled by two factors, chronological age (weeks) and physiological age (body weight) (Renema *et al.*, 1999a; Applegate, 2001). Body weight is important as it affects attainment of the body composition necessary to support sexual maturity (Brody *et al.*, 1980; Soller *et al.*, 1984; Dunn *et al.*, 1990); consequently, birds with light body weight will have delayed attainment of sexual maturity. Age also plays a key role in initiating the reproductive cycle since maturation of the hypothalamus (its capability to respond to photoperiod and hormone feedback) is correlated to age rather than body weight (Robinson *et al.*, 2003). Thus, each body component must reach a sufficient stage of development to enable it to perform its physiological role in supporting the whole body processes associated with sexual maturity. Katanbaf *et al.* (1989b) suggested that

attainment of sexual maturity seems to depend mostly on age (capability of the hypothalamus to perform its role) in broiler breeders fed *ad libitum*, whereas in restricted fed birds body weight and carcass composition are more important.

1.4 Effect of nutrition on reproduction

Current broiler breeder breeds are, beyond doubt, the successful result of over fifty years of intensive genetic selection for rapid growth. Their offspring weigh about 50g at hatch and reach around 2kg within 37 days (Weeks, 2004). This is accompanied by a reduction in the age at which they reach market weight of a half-a-day (Havenstein *et al.*, 2003a) to one day (Gyles, 1989) each year. It was reported by Marks, (1980) that this increase in growth rate is related to the propensity to increased feed intake (Barbato, 1994; Richards, 2003) and this was due to the increased appetite in broiler strains as demonstrated by Denbow, (1994). Subsequent observations of Hocking *et al.* (1993) demonstrated that the breeder birds spend most of their time eating and resting and, even during the lights off period, they spend a considerable proportion of time searching for feed thus indicating that their appetite cannot be satisfied. These modern strains are expected to have the genetic potential for rapid growth, in addition to good reproductive characteristics to produce the generation of market chickens. A negative relationship was identified between these two variables (Jaap & Muir, 1968; Nestor *et al.*, 1980), so broiler breeders are feed-restricted.

In summary, geneticists have successfully developed broiler breeders that possess the genetics for fast growth, which will be inherited by their

offspring. However, the pronounced result of such genetic selection was feed over consumption, which is favourable for their progeny as they are not raised to reach sexual maturity, but has negative impacts on the breeders themselves. Impairment to reproductive wellbeing has been addressed as growth and reproduction move in opposite directions. Thus, feed restriction was implemented in order to minimize these negative outcomes with the primary aim of determining the most efficient age to feed-restrict birds. Some of the genetic selection outcomes are outlined in Table 1.3.

Table 1.3 Some correlated responses to genetic selection for increased juvenile growth in chickens (Siegel & Wolford, 2003)

Trait	Response
Adult body weight	+
Feed efficiency	+
Feed consumption	+
Plasma growth hormone	-
Carcass fat	+
Immunocopetence	-
Muscle number	+
Muscle size	+
Yield	+
Egg production	-
Egg size	+

1.4.1 Effects of *ad libitum* feeding

The first notable effect of *ad libitum* feeding is obesity (Chambers *et al.*, 1981; Havenstein *et al.*, 2003a, b) and many undesirable outcomes that are collaterally resulting from this condition. Thus, broiler breeders are prone to lameness and leg deformities (Hester, 1994; Julian, 1998; Butterworth, 1999; Sanotra *et al.*, 2001), fatty liver and kidney syndrome (Whitehead *et al.*, 1978), and ascites syndrome (Julian, 1993; 1994; Wideman, 2000; Scheele *et al*; 2003). In addition, obesity might lead to an inefficient mating

resulting in reduced fertility (Bilgili & Renden, 1985), hatchability and embryonic viability (Yu *et al.*, 1992b), and consequently reduced chick production. Another possible cause of reduced hatchability is the egg sequence length, which is shorter in *ad libitum* compared to restricted-fed females resulting in increased number of the first sequence eggs; these are particularly subject to embryonic death due to the fact that they are held over the pause day, resulting in shell defects that may also play an important role in reducing hatchability (Renema *et al* 1999c). A second point is the higher incidence of erratic laying in short egg sequences which is correlated with poor shell formation (Robinson *et al.*, 1991b; Fasenko *et al.*, 1992).

Differences between *ad libitum* and restricted feeding were examined by Hocking *et al.* (2002a) and are outlined in Table 1.4.

Table 1.4 Productivity and mortality to 60 weeks of age in feed restricted and *ad libitum* fed broiler breeder females (Hocking *et al.*, 2002a)

Trait	Restricted	*Ad libitum*
Final body weight/kg	3.7	5.3
Mortality %	4	46
Egg (number/hen)	157	51
Hatching eggs (number/hen)	140	40
Egg weight/g	65	65
Fertility %	86	87
Hatchability %	86	43

It was found by several groups (Bornstein *et al.*, 1984; Robbins *et al.*, 1986; Robinson *et al* 1991a; Yu *et al.*, 1992a, b) that, although females fed *ad libitum* start laying eggs early (as it accelerates the sexual maturation process; Wilson & Harms, 1986) compared to their feed restricted counterparts, an earlier decline in egg production occurred, therefore their egg production tends to be lower in total, whereas feed restriction seemed to

cause better persistence and longer sequences (Fattori *et al.*, 1991). This reduction in total egg numbers, in spite of the existence of many large yellow follicles, was studied by Hocking *et al.* (1987) and Renema *et al.* (1999b) who found an increased incidence of follicular atresia (follicles of less than 5mm diameter) in restricted versus females fed *ad libitum* leading to pronounced alteration in reproductive hormones which, in turn, affected ovarian development. *Ad libitum* feeding caused a significant increase in the number of large yellow follicles arranged in multiple hierarchies (Hocking *et al.*, 1987, 1988, Hocking; 1992, Renema *et al.*, 1998), with an elevated rate of unsettable eggs (Jaap & Muir, 1968) and double-yolked eggs, attributed to laying more than one egg per day (Renema *et al.*, 1999c), thin-shelled or abnormal shell (Katanbaf *et al.*, 1989) in addition to high incidence of internal ovulations (Udale *et al.*, 1972) and also laying eggs outside the normal laying period (Yu *et al.*, 1992b). LYF number is significantly correlated to body weight (Hocking and Whitehead, 1990). Moreover, Robinson *et al.* (1991a) illustrated that a significant proportion of the body weight increase was made up of fat, leading to concerns that the deposition of that excess fat in non-fat cells culminated in dysfunction of those cells, as observed in the pancreas by Unger and Orci (2000).

1.4.2 Timing of feed restriction

Growth-selected strains seem to be more sensitive to overfeeding through the sexual maturation process period and this is inversely dependent on age; the nearer the sexual maturity, the greater the sensitivity (McGovern *et al.*, 1997).

Many studies have been undertaken in order to specify the threshold time for feed restriction, when it can improve reproductive performance efficiently (McDaniel *et al.*, 1981; Bornstein & Lev, 1982; Robbins *et al.*, 1988; Bruggeman *et al.*, 1997, 1998a); some of these researchers suggested the importance of restriction throughout the rearing period, whereas others claimed that there should be a critical time during the rearing period. Bruggeman *et al.* (1999) conducted an experiment where birds were restricted at different ages during rearing period until the first egg was laid; thereafter the birds were fed *ad libitum* until the end of lay as illustrated in Figure 1.5. Results presented in Table 1.5 showed that restriction from week seven to fifteen had the best egg production and settable eggs values; therefore it was suggested that there was no need for longer restriction. In addition, Cerci *et al.* (2003) did not find differences between *ad libtum* and restricted birds, when restricted from 15-18 wks of age.

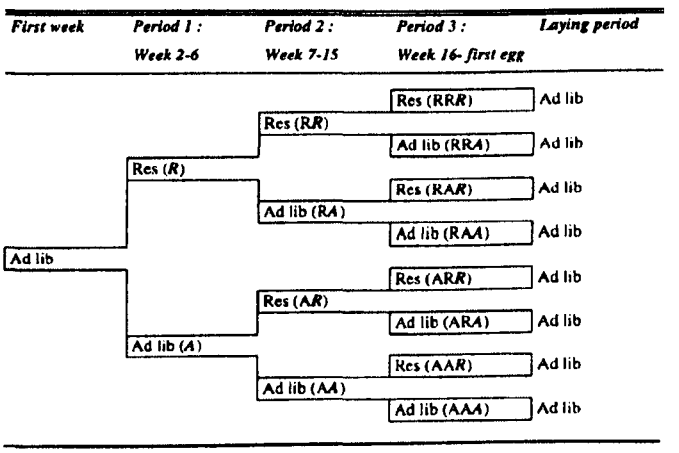


Figure 1.5 Feeding groups during rearing period (Bruggeman *et al.*, 1999)

Table 1.5 Cumulative egg production (total and settable) from the onset of lay to 50 week of age (Bruggeman *et al.*, 1999)

Feeding regimen							
RRR	RRA	RAR	RAA	ARR	ARA	AAR	AAA
Cumulative egg production/bird							
108.2±2.68^{b}	111.0±2.72^{b}	91.5±4.13^{c}	94.9±4.19^{c}	113.6±3.01^{b}	124.4±3.67^{a}	108.6±3.58^{b}	83.1±3.51^{d}
Cumulative settable eggs/bird							
91.2±2.92^{b}	92.7±2.72^{b}	96.8±3.88^{d}	80.4±3.63^{c}	96.1±3.26^{ab}	103.4±3.40^{a}	83.3±3.07^{c}	69.6±3.24^{d}

a-d Means within arrow lacking common superscript differ (P< 0.05)
values are expressed as means ± SEM (n=8)
R=Restricted feeding; A= *Ad libitum* access to feed.

In summary, restricted feeding has been shown to improve breeders' reproductive performance; however determination of the best time for feed-restriction was the concern of various researchers, who have conducted many experiments on that purpose in order to balance between breeders' reproductive performance and feed-satiety.

1.4.3 Feed restriction and animal welfare

Animal welfare has been the focus of much attention in recent years. Although restricted feeding has positive effects on both health and reproduction, on the other hand there is a wealth of scientific evidence on its negative effects on animal welfare. Feed restriction resulted in considerable stress and abnormal behaviour which is indicative of frustration of feeding motivation, aggressiveness in males (Mench, 1988), fearfulness in females (Savory *et al.*, 1992), and over-drinking (Kostal *et al.*, 1992; Hocking, 1992). However, Millman and Duncan, (2000a) indicated that feed-restricted males showed less aggression compared with males fed *ad libtum*.

It is evident that *ad libtum* feeding markedly interrupted the balance between growth and reproductive wellbeing in contrast to restricted feeding,

which showed efficient performance in terms of reproductive parameters. Therefore, it can be said that feed restriction may have less effect on birds' welfare, although alternative choices were suggested by Bruggeman *et al.* (2005). Two choices were suggested in order to ally growth requirement with good reproductive performance, health and welfare. The first is continuing the current genetic selection, and in this case restriction should be minimized to the lowest effective duration, or diets should be modified to contain less energy; thus birds are energy-restricted rather than feed-restricted. The second choice is changing the aims of genetic selection in order to produce new lines that can tolerate *ad libitum* feeding such as slower-growing Label chickens (France); dwarf breeders could be another alternative, as possessing the dwarf gene is suggested to reduce the necessity for feed restriction. As Hocking *et al.* (1987) showed, normal broiler breeders and dwarf broiler breeders did not differ significantly in their reproductive performance. Additionally, results of a study undertaken on dwarf broiler breeders have shown that reducing feed allowance during the laying period depressed egg production (Triyuwanta *et al.*, 1992).

1.5 Photoperiodism

Day length is considered to be the predominant environmental cue used by avians to stimulate proper reproductive responses (Baker, 1938; Wingfield & Kenagy, 1991 cited by Ball & Ketterson, 2008; Murton & Westwood, 1977).

1.5.1 Perception of light

An interesting feature in birds is that, although both eyes and the pineal gland have sensory receptors that perceive light leading ultimately to the production of melatonin (Zimmerman & Menaker, 1979; Binkley *et al.*, 1980; Hamm & Menaker, 1980; Takahashi & Menaker, 1984; Underwood & Siopes, 1984), they are not essential in the reproductive process (Benoit, 1935 cited by Dawson *et al.*, 2001; Wilson, 1991). Subsequent studies have led to the demonstration that photoreceptors, located deep in the hypothalamus, mediate the response to the photoperiodic cue and transmission of photon energy into neural impulses. This, in turn, will be amplified by the anterior pituitary that is deep in the brain near the hypothalamus (Menaker *et al.*, 1970; Yokoyama *et al.*, 1978; Foster *et al.*, 1985). In spite of intensive studies to identify the exact location of these photoreceptors (Silver *et al.*, 1988; Saldanha *et al.*, 1994), they have yet to be definitively identified (Ball & Ketterson, 2008).

1.5.2 Critical and saturation day length

Critical day length referrers to the minimum photoperiod required to stimulate gonad responses. Conversely, the maximum day length leading to the maximal reproductive response is defined as the saturation day length (Sharp, 1984; Rose, 1997; Robinson, 2003). Birds are not stimulated when day length is below the critical day length; once the day length reaches the critical point (11 hours) then the hypothalamic-pituitary-ovarian axis is stimulated. This photo-response continues with increasing day length up to between 14 and 17 hours, when birds are said to be photoperiodic-saturated

and become photorefractory. The time during which birds respond to increasing day length is termed marginal day length.

1.5.3 Circadian rhythms and photosensitive phase

Birds utilize dawn or the time when the light is turned on to set their internal clock to 0; approximately 11-13 hours later the photosensitive period will commence. It was suggested by Ball and Balthazart (2003) that two signals of light are sufficiently effective to activate photoperiodic responses in avians; the first is to set the internal photoperiodic clock that will determine the photosensitive phase during which the second pulse must occur that will be recorded by the hypothalamic photoreceptors which will culminate in the hormonal cascade. The main elements involved in the reproductive process are presented in Figure 1.6.

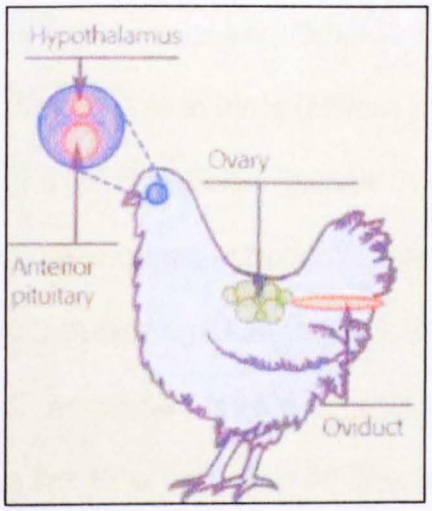


Figure 1.6 The main reproductive organs of the laying hen (Robinson *et al*, 2003)

1.5.4 Photostimulation and initiation of reproduction

During short photoperiods, when day length is still below the critical day length (winter and early spring), concentrations of gonadotrophin releasing hormone (GnRH) are low. As day length increases and reaches the critical day length, photoreceptors in the hypothalamus will be activated and transform photon energy into neural pulses stimulating the secretion of the neuropeptide luteinizing hormone releasing hormone (LHRH) which, in turn, will be transported in the blood stream to the anterior pituitary and stimulate production and secretion of the gonadotrophins luteinizing hormone (LH) and follicle stimulating hormone (FSH) which act at the testicular or ovarian level resulting in the maturation of the gonads and production of sperm and follicles, and thus stimulate the onset of lay (Dunn & Sharp, 1990; Lewis *et al.*, 1999). Two types of GnRH, GnRH-I & GnRH-II, are present in the avian brain (Ball & Balthazart, 2003). However, GnRH-I seems to be the principal regulator of gonadotrophin release in birds (Millam *et al.*, 1993).

In the ovary, preovulatory follicles (except the largest follicle) produce steroid hormones (androgens and oestrogens; Senior, 1974) that will feed back to the hypothalamus to aid regulation of sex hormone levels in order to prompt development of secondary sexual characteristics resulting in the transformation of the pullet to a hen. Ten to eleven days after initiation of puberty, the first egg will be laid. However, this response to increasing day length will continue until it reaches the saturation day length (14 to 17h); thereafter the bird will no longer exhibit any response, termed as photorefractoriness, which is also a result of long day length. Dissipation and breakdown of this photorefractoriness is attained when birds are subject to

short day length in autumn (Follet *et al.* 1977; Etches & Duke, 1984; Etches, 1990; Yu *et al.*, 1992; Rose, 1997). Thus, it is worth considering the importance of experiencing short days to reset the bird's sensitivity to the stimulatory photoperiodic cue (Ball & Ketterson, 2008).

Robinson *et al.* (1996) recommended photostimulating birds at later ages (19 to 21 wks) in order to lessen differences in the rate of sexual maturity between birds. Although several weeks separate photostimulation and onset of lay, increases in LH and FSH levels could be detected during the night of the first day of photostimulation, which can be considered a sign that the HPG axis has been activated (Rose, 1997).

Holberton and Able (1999) tested the effect of using dim light rather than a constant photoperiod; results showed that gonadal growth, regression and moult occurred, however over a considerably greater time. Previous investigations showed that eggs can be laid in the absence of dark/light cycles, in which birds depend on other cues, such as noise, temperature, feeding cycle, to set the circadian rhythm for LH release; however, in this case eggs will be laid throughout the day (Cain & Wilson, 1974; Yoo *et al.*, 1986; Bhatti, 1987).

1.5.5 The open period of LH

The progesterone secreted by the largest preovulatory follicle stimulates the LH surge, which is restricted to 6 to 8 hours, defined as the LH open period which leads to the release of the largest follicle.

The primary source of the progesterone causing the first increase in LH level is the largest five hierarchal follicles, which will stimulate further

release of progesterone from the largest follicle (Etches, 1984; Verheyen *et al.*, 1987).

This surge occurs on a daily basis; it is also a light signal used to set the daily clock of the LH open period which is at dusk or the lights off. If the follicle is not mature enough during the open period, it will stay until the next preovulatory LH surge resulting in a day pause and starting a new egg sequence (Etches, 1996).

Collectively, light is considered the key regulator of the reproductive process. It plays a dual role; the first sets the beginning of the reproductive cycle, once birds detect the efficient day length (critical day length) at which photon energy is translated to neural output, activating the HPO axis resulting in the onset of lay. The second regulates the daily ovulatory cycle, as the release of the largest follicle must occur during the LH open period, which is set to zero at dusk or when the light is turned off, and is effective for 6-8 hours.

1.6 Follicle structure and development

1.6.1 Follicle structure

Shortly after hatch, cells originating from the mesoderm surround the primordial germ cells; these develop during the embryonic period and, in turn, will develop into two types of functionally different cells: granulosa cells (inside); and theca cells (outside) and are separated by the basement membrane (basal lamina). Theca cells are a mixture of types; one type lying immediately next to the basement membrane that is believed to be the steroid producers, and predominant outer cells supporting theca tissue

(Herman *et al.*, 1998; Etches, 1990; Johnson, 2000). The follicle is supplied with blood flow through vessels branched from the ovarian artery, with greatest supply being to the hierarchal follicles (Etches, 1990) with fine arterioles passing through theca tissue to the basal lamina carrying the precursors needed for yolk formation (Scanes *et al.*, 1982). The follicle is also innervated, with the greater number of neurons being provided to the hierarchal follicles, supplying the neurochemicals important for organizing the follicle development process (Gilbert, 1969; Dahl, 1970; Unsicker *et al.*, 1983).

1.6.2 Follicle development

Follicle growth is a process of four stages; during the first stage, recruitment of granulosa cells occurs which, in turn, form the peri-vitelline layer next to vitelline membrane that surrounds and encloses the primary oocyte resulting in the formation of the primodial follicles of 80μ in diameter, at this stage the early growth of the primary follicles commences, where the theca layer is formed with basal lamina separating it from granulosa cells, diameter of primary follicles ranges between 0.08 and1mm and they may persist for years. Thereafter, primary follicles enter the slow growth phase where white yolk is accumulated over a period of time ranging from weeks to months resulting in the prehierarchal follicles formation (2 to 8mm diameter), which is well innervated and vascularised; at this stage the theca interna and externa are clearly distinguished. Subsequently, a single prehierarchal follicle is selected to enter the rapid growth phase. At this final stage granulosa cells are reorganized into a monolayer, as they were initially multi-cell layer, and

they become squamous in shape rather than cuboidal except in the germinal disk region (Gilbert *et al.*, 1980; Johnson & Woods, 2007) giving more spaces and less cell junctions allowing more supply with yolk material (Shen *et al.*, 1993); for a few days, the follicle grows from 9mm to around 40mm when it becomes ready for ovulation (Channing *et al.*, 1980; Kovacs *et al.*, 1992) (Figure 1.7).

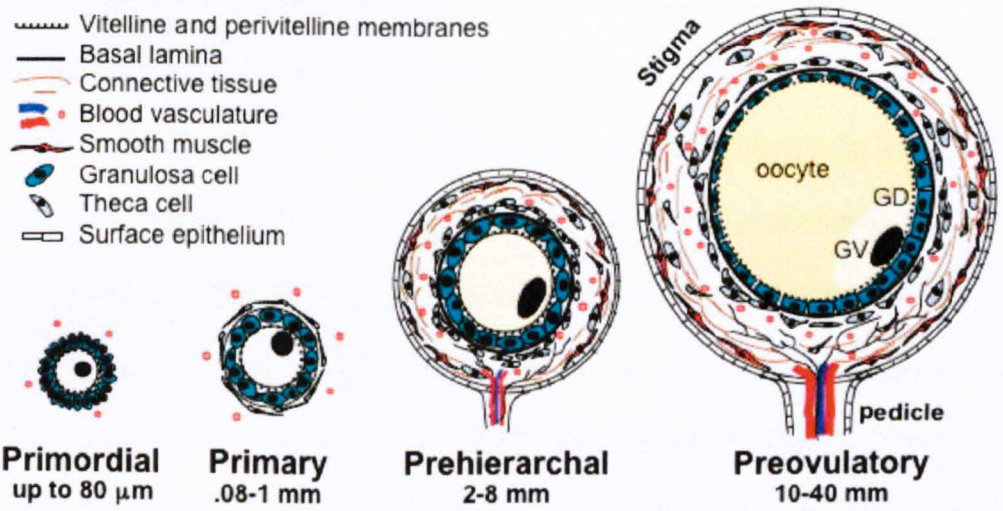


Figure 1.7 Growth and development of follicles in the avian ovary. GD: germinal disk, GV: germinal vesicle (Johnson & Woods, 2007)

1.6.3 Follicle selection into the preovulatory hierarchy (cyclic recruitment)

In the reproductively active ovary, a hierarchy of five to eight follicles represents the follicles that will ovulate (Bahr & Johnson, 1984; Etches & Petitte, 1990), in addition to the presence of a cohort of 6-8mm diameter follicles, which are considered of high importance in maintaining the follicular hierarchy as it is the source from which a follicle will be chosen into the hierarchy on a daily basis (Evans, 2003; Johnson *et al.*, 2004; Johnson & Woods, 2009) that, however, need not necessarily be the largest within the

cohort (Woods & Johnson, 2005). Granulosa cells in both types of follicles have different features; in prehierarchal follicles granulosa cells are undifferentiated with dominance of follicle stimulating hormone receptor expression (FSHR), and trivial luteinizing hormone receptor (LHR) expression. They are mitotically active, in addition to their inability to produce progesterone (Tilly *et al.*, 1991a) which might be related to the inactivation of P450 cholesterol side chain cleavage (P450scc) enzyme (Tilly *et al.*, 1991b). In contrast, preovulatory granulosa cells are highly differentiated exhibiting high level of LHR, P450scc enzyme activity, steroidogenic acute regulatory (StAR) protein. This protein is believed to enable cholesterol transfer to the inner mitochondrial membrane where it is converted to pregnenolone by the mediation of P450scc enzyme (Johnson *et al.*, 1996; Johnson *et al.*, 2001; Stocco, 2001; Johnson *et al.*, 2002; Woods *et al.*, 2007). Thus, granulosa cells are capable of synthesising and secretion of appreciable amounts of progesterone in response to LH, a prerequisite for ovulation (Huang *et al.*, 1979; Lang *et al.*, 1984; Johnson & Tienhoven, 1984). Proliferation of granulosa cells occurs in this type of follicle, however, at a low level and not at the non-germinal disk region (GDR) (Perry *et al.*, 1978; Tilly *et al.*, 1992a; Etches, 1996). As many researchers have recognized, there are morphological and functional differences between granulosa cells (GCs) *per se* in relation to their location relative to the germinal disc, with high differentiation and maximal progesterone production given to the distal cells, compared to GCs in the GDR which are undifferentiated and mitotically active, whereas proximal GCs are intermediate in progesterone production (Marrone *et al.*, 1990; Tischkau & Bahr, 1996; Yao & Bahr, 2001).

Many factors seem to play role in proliferation of the prehierarchal follicles. Johnson *et al.* (1993) postulated that increased proliferation in prehierarchal granulosa cells is accompanied by a high presence of mRNA for the nuclear transcription factor c-Myc; nuclear phosphoproteins, which mediates normal cell proliferation (Blackwood & Eiseman, 1991; Eilers *et al.*, 1991) in addition to its role inhibiting cell differentiation (Freytag *et al.*, 1990). Epidermal growth factor (EGF) and transforming growth factor alpha (TGFα) have an important role promoting elevated c-Myc mRNA expression, thus stimulating granulosa cell proliferation (Johnson, 1993); the latter was also found to inhibit cell differentiation via stimulating mitogen-activated protein kinase (MAPK) which blocks StAR expression (Johnson *et al.*, 2002). EGFs were also shown to inhibit cell differentiation, however in different pathways (Woods *et al.*, 2007). Enhanced FSHR mRNA, which leads to enhanced formation of cyclic adenosine monophosphate (cAMP) which, in turn, is considered to have an appreciable role in the initiation of LHR and progesterone production, are concomitant with alleviation of the inhibitory effect of MAPK (Li & Johnson, 1993; Hernandes & Bahr, 2003; Woods & Johnson, 2005); however, the mechanism is still obscure (Johnson & Woods, 2009).

Although Woods *et al.* (2007) suggested that follicles of 9-12mm in diameter represent the initial distinguishable stage following follicle selection, as yet the pivotal signal which determines prehierarchal follicle selection seems to be far from clear.

1.6.4 Postovulatory follicles (POFs)

The follicle is the sac containing the ovum and attaches it to the ovary. As ovulation occurs the follicles, consisting of granulosa and theca cells, will remain for 6to10 days (Johnson, 2000) after which they regress via apoptosis (Tilly *et al.*, 1991b), as they exhibit low rate of activity (Chalana & Guraya, 1978) as well as receiving lower blood supply (Robinson *et al.*, 2003). However the possibility of its participation in oviposition was addressed by Kelly *et al.* (1990) as ligation blocked oviposition.

1.6.5 Follicle atresia

Throughout postnatal life of the female, out of thousands of follicles present at hatch, 250-500 follicles develop and are ovulated (Johnson, 2000), whereas more than 0.99 of follicles degenerate through attrition (Parshad, 1997; Johnson, 2003). Atresia is most likely to occur in prehierarchal follicles of less than 9mm in diameter; atresia among preovulatory follicles is very limited (Gilbert *et al.*, 1983) (Figure 7).

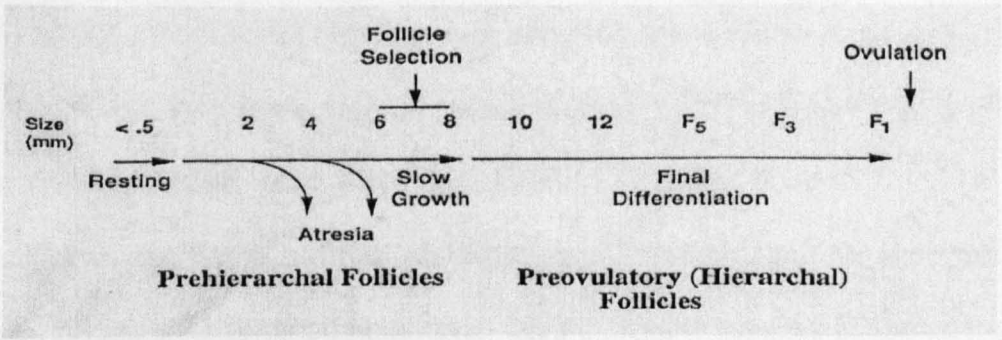


Figure 1.8 Ovarian follicle hierarchy (Johnson, 1996)

Evan *et al.* (1992) suggested that mitotically active cells are more vulnerable to apoptosis than non-mitotic cells, as active mitosis might be an

essential condition for apoptosis instigation. In relation to this, Yushimura *et al.* (1994) provided results regarding paracrine signals being released by the germinal disc region (GDR) to the outer granulosa layer retaining its viability, and that any destruction to the outer granulosa layer leads to preovulatory follicle atresia.

The particular type of programmed cell death (PCD) apoptosis is the process through which atresia occurs, and is instigated within granulosa cells (Tilly *et al.*, 1991c; Johnson *et al.*, 1996b; Tilly, 1997; Jiang *et al.*, 2003). Thereafter, the cell death message is most probably transmitted via gap junctional coupling (Krysko *et al.*, 2004), in that apoptosis of granulosa cells might be considered the earliest indicator of the onset of atresia (Johnson *et al.*, 1996b).

Many factors might play a dual role either in cell viability or cell death. These factors might be proteins, enzymes or genes, such as Bcl-2- related family of regulatory proteins , interleukin converting enzyme related family of enzymes, in addition to a selected group of protooncogenes (such as, c-myc) and tumour suppressor genes (Johnson, 2000; 2003).

During embryonic development gametes accumulate in the left gonad. By the time of hatching oogenesis is terminated, with the number of gametes that the female will ever have throughout life being established. Prior to sexual maturity, the pullet has an undeveloped ovary, however within a few weeks after photostimulation the hypothalamic-pituitary-ovarian communication is activated leading to ovarian development. Thus, recruitment of follicles from the pool of small follicles is commenced and

thereby the mature ovary is arranged in an obvious hierarchy consisting of follicles at different stages of development.

The recruitment of a single follicle from a cohort of 6-8mm diameter follicles to enter the rapid growth phase occurs on a daily basis; however the cause for a specific prehierarchal follicle to be selected is still to be determined. Following follicle selection, granulosa cells undergo morphological changes and become differentiated and capable of steroid production. Follicular development and maturation is regulated by pituitary hormones (LH and FSH), in addition to a group of factors termed growth factors, such as the epidermal growth factor family and transforming growth-β superfamily, which are believed to inhibit or promote gonadotrophins according to stage of development, in order to maintain harmonious steroidogenesis. These factors have been identified in the granulosa and/or theca cells and act in autocrine, endocrine or paracrine manners to exert their effect.

The growing follicles (prehierarchal follicles) are, commonly, more prone to undergo atresia compared to hierarchal follicles (preovulatory follicles). This might be due to apoptopic resistance by granulosa cells which they gain as they become differentiated. It is well documented that cell death is initiated within the granulosa cell layer then in the other cell compartments. Many factors participate in organising this physiological process, such as some groups of proteins, enzymes and genes. Finally, hierarchal follicles become vulnerable to undergo atresia as the hen ages.

1.7 Yolk components

Accelerated accumulation of yolk material commences as the follicle enters the rapid growth phase 7-10 days prior to its ovulation, during which its diameter reaches 35-40mm (Etches, 1996). The components of yolk material (g/kg) are: 326 lipids, 160 proteins, 10 carbohydrates and 11 minerals (Romanoff & Romanoff, 1949 cited by Johnson, 2000). As the yolk content of lipids is double its protein content (Robinson *et al.*, 2003), therefore, it seems appropriate to investigate lipids in terms of their importance and function, major classes and distribution throughout the body as these lipids are the main supplier of precursors that will be used for yolk formation.

1.7.1 Lipid classes and importance

The major lipid classes present in the yolk are: triacylglycerol (TAG) accounting for approximately 0.67, phospholipid (PL) 0.25, free cholesterol (FC) 0.05. Cholesterol esters (CE) and free fatty acids (FFA) are found in trace amount (Speake *et al.*, 1998). Lipids, in various classes, possess several important functions throughout body tissues. Triacylglycerols are the major energy supplier needed for embryo development, owing to their significant content of fatty acids. Phospholipids serve as an essential component of cell membrane structures (Speake *et al.*, 1998). Sterols are also of importance as integral components of cell membranes, as well as specific sterols being hormones; sex hormones and adrenocortical steroids. Glycolipids act as structural entities in the central and peripheral nervous system (Stevens, 1996). Lipids in general are also important in body

insulation (Rose, 1997). The molecular structure of the different lipid classes is outlined in Figure 1.9.

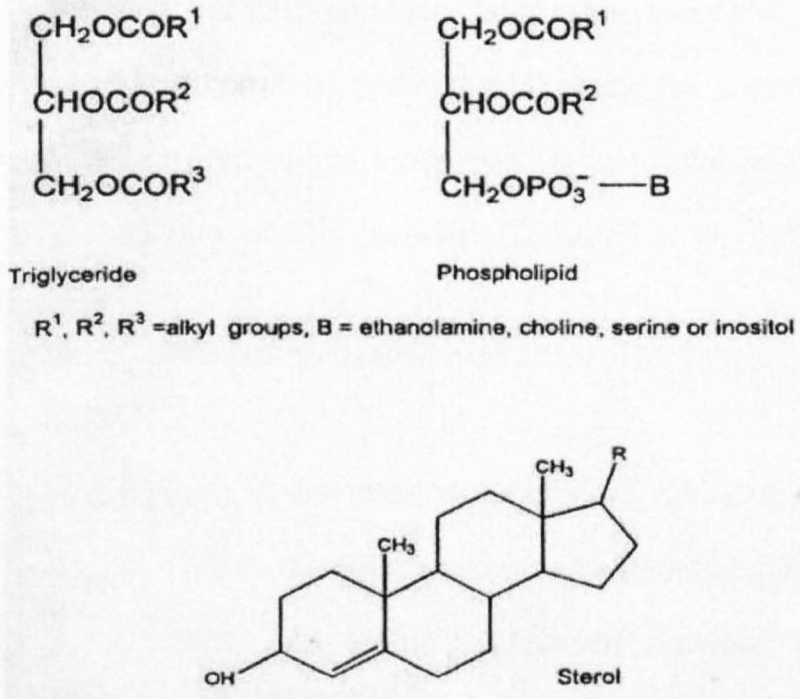


Figure 1.9 Structure of typical triglycerides, phospholipids and sterols (Stevens, 1996)

1.7.2 Lipid distribution

Lipid accumulation commences as early as 12-16 days of embryonic development, during which yolk lipid is transferred and deposited in adipocytes (Noble & Cocchi, 1989; 1990; Stevens, 1996). Although lipid is present as a droplet in several body cells, droplet diameter rarely exceeds 2μm, in contrast to adiopcytes in which lipid droplets might be as large as 100 μm (Evans, 1977). In fact, new adipocytes are formed at early stages of life (hyperplasia), whereas the increase in the droplet size (hypertrophy) commonly occurs as the bird approaches its mature body size (Rose, 1997). Adipose tissue, a matrix of round or oval cells defined as adypocytes in which triglycerids are stored, consists of 0.80 fat and 0.20 water with a small

amount of protein, and is found either in the body cavity or under the skin. Body cavity fat is found either in the upper part of the body cavity surrounding the digestive tract and kidneys or in the lower part close to the cloaca forming what is known as abdominal fat or leaf fat. Subcutaneous fat, found under the skin, represents the second type of adipose tissue. Both types comprise the bulk of lipid reserves (Cahaner *et al.*, 1986; Stevens, 1996; Rose, 1997).

1.7.3 Yolk spheres

Four units, varying in diameter and content of lipid and protein, form the tightly packed 140μm diameter yellow yolk spheres, particles, sub-droplets, lamellar bodies and a finely dispersed aqueous phase. The particles of 27nm diameter possess the highest content of lipid, consisting of 0.95 of yolk lipids (0.65 of yolk solids) contain the very low density lipoprotein (VLDL) of density less than 1.006 g/ml (Swift, 1995), which consists of 0.88 lipid and 0.12 protein. Sub-droplets are based on vitellogenin, a structure of 100-2500nm diameter containing 0.15-0.20 lipid and 0.23 protein in the form of two phosvitin and two lipovitellin molecules. Lamellar bodies exist in membrane- like form with 100-200nm diameter, and finally the aqueous phase which contains the lowest amount of yolk solids (0.10) which are traces of lipids but mostly proteins (Etches, 1996).

1.7.4 Lipid biosynthesis

Birds in general are capable of lipid synthesis and two valuable points address this capability; the first is the increase in the total body lipid throughout the first six weeks of age, during which lipid content doubles

every 5.5 days (Scanes, 1987). The second is the egg-laying period during which birds can produce an egg with 6g of lipid content whereas the diet derived lipid does not exceed 3g (Griminger, 1986). The liver is the main site of lipid biosynthesis, rather than adipose tissue, and most of the liver fat content is dietary derived (Griminger, 1986; Griffin & Hermier, 1988). Hepatic lipid synthesis is stimulated by elevated concentration of oestrogen at the onset of ovulation (Nimpf & Schneider, 1991).

1.7.5 Fatty acid metabolism

Long chain fatty acids in the liver are derived from three sources, adipose tissue (non-esterified fatty acids), diet (hydrolysis of dietary TAG) and liver (synthesis from acetyl-CoA resulting from excess glucose breakdown). Once these fatty acids enter the liver, they might be esterified into TAG or, less importantly, to phospholipids (PL) and cholesteryl esters (CE), or they might be oxidised either completely to CO_2 during synthesis of ATP molecules, or incompletely to acetate and ketone bodies (Zammit, 1990; Gruffat *et al.*, 1996). Many factors might determine the fate of these long chain fatty acids, such as nutritional status (of major importance), chain length and degree of saturation, and hormones (McGarry & Foster, 1980, Rustan *et al.*, 1992; Geelen *et al.*, 1995; Zammit, 1995).

1.7.6 Fatty acids biosynthesis

Fatty acid biosynthesis is a process of seven steps starting from acetyl-CoA; in each of the first six steps, two carbon atoms will be added to the expanding fatty acid chain; the seventh step results in release of the free fatty acid. This process is mediated by two enzyme systems: acetyl-CoA

carboxylase and fatty acid synthetase which is a multi-enzyme polypeptide. The pathway is outlined in Figure 1.10.

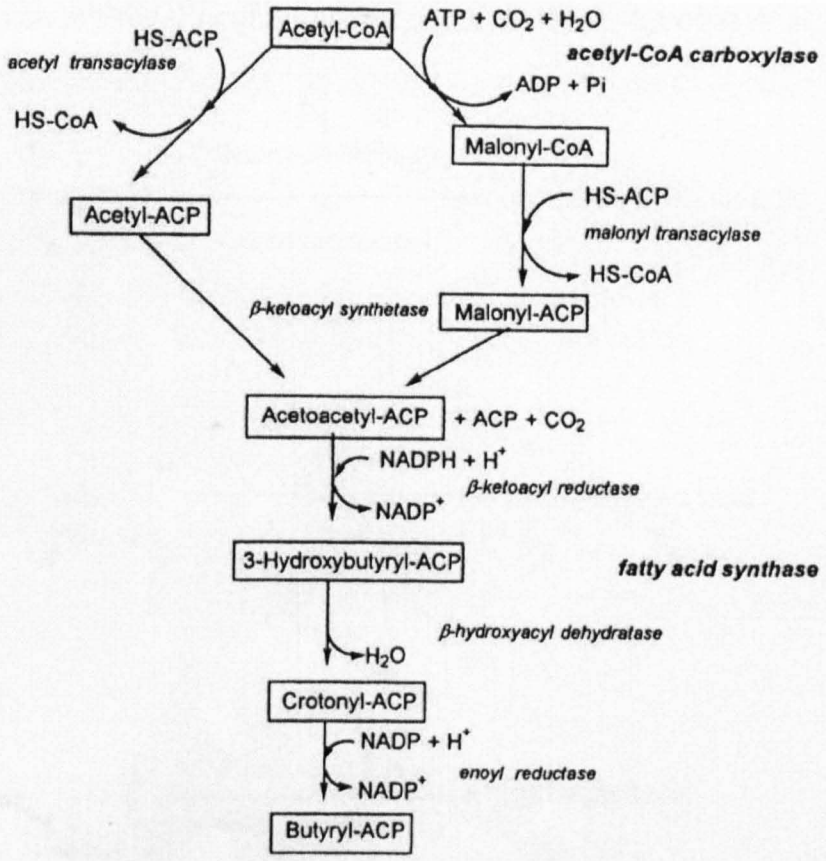


Figure 1.10 Biosynthesis of fatty acids (Stevens, 1996). The resulting butyryl-ACP will condense with another malonyl-ACP molecule and after 7 rounds of this cycle palmitic acid is produced.

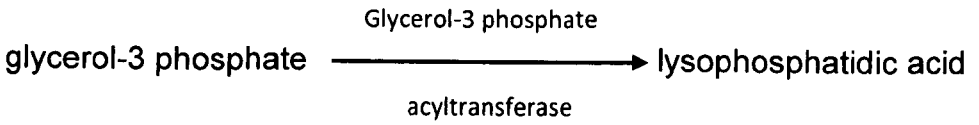
1.7.7 Triacylglycerol (TAG)

Triacylglycerols are the major component of yolk lipids (Bujo *et al.*, 1995). The structural feature of TAG is the glycerol moiety esterified by three carboxylic acids that might be the same or different. Carbon chain length, degree of saturation and position of the double bonds are all key features of fatty acids and confer on TAG a variety of physical properties (Stevens 1996; Sato *et al.*, 2005). Palmitic (C:16) and oleic (C:18) acids are the most common components of TAG (Burley & Vadehra, 1989; Yao *et al.*, 1991).

1.7.8 Triacylglycerol synthesis

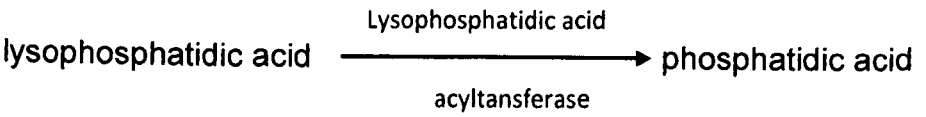
Hepatic TAG synthesis occurs through a process of four reactions, three of which are esterification and one is hydrolysis. (Gruffat *et al.*, 1996)

1.



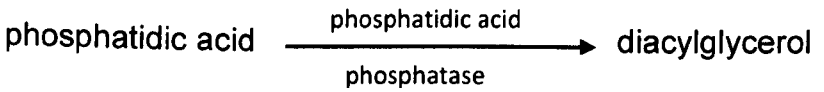
Endoplasmic reticulum (**ER**) or the mitochondria

2.



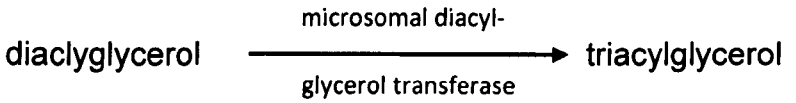
ER

3.



ER

4.



1.7.9 Lipid transport

Lipoproteins are structures where protein is associated with lipids to transfer them from their site of synthesis to the peripheral tissue where they are utilised, and *vice versa*. Protein serves two crucial functions in this structure: to solubilise lipids and to bind to the proper tissue receptors (Brown & Goldstein, 1986; Stevens, 1996). Four forms of lipoproteins are available to play this role:

1. Very low density lipoprotein (VLDL that transports mainly TAG)

2. Low density lipoprotein (LDL, that transports cholesterol, and are believed to be the cholesterol and cholesterol esters rich in the remaining part of VLDL after TAG have been lost)

3. High density lipoproteins (HDL, responsible for cholesterol transport from the peripheral tissues to the liver where it will be degraded)

4. Vitellogenin (lipophosphoproteins)

1.7.10 Very low density lipoprotein (VLDL)

The main components of VLDL are lipids (0.88) and proteins (0.12). The lipid part consists of TAG (0.70-0.75), phospholipids (0.20-0.25), and cholesterol (0.04). Protein components are apolipoprotein-B (1 molecule) and apoVLDL-II (25 molecules) (Etches, 1996). These two proteins possess unique properties, which ensure secure transfer of TAG to the developing oocyte. ApoB is the protein responsible for binding to specific receptors, 95kDa which only can recognise specific regions of apoB, embedded on the vitelline membrane. Apo-II has two features; the first confers on VLDL resistance to lipoprotein lipase enzyme activity (Schneider *et al.*, 1990), thus securing high amounts of TAG for the egg yolk (Griffin *et al.*, 1982). The second feature is reduction of VLDL particle size from 55-60nm to 27-35nm, after attachment of Apo-II to VLDL, enabling VLDL to pass through the basal lamina membrane which acts as a filter allowing a selected size to access the oocyte (Griffin & Perry, 1985; Etches, 1996; Walzem, 1999). The vitelline membrane has a valuable role in increasing upload of VLDL via its projections into the developing yolk, giving more surface area and

consequently increased receptor-mediated endocytosis (Griffin, 1992; Schneider, 1995; Etches, 1996; Bujo *et al.*, 1997).

Exogenous TAG absorbed in the small intestine are transported to the liver via the blood stream as lipoprotein particles, defined as portomicrons which, as a result of their larger size, are prevented from passing through the basal lamina membrane (Stevens, 1996), and are re-esterified (Wiggins & Gibbons, 1992). Figure 1.11 outlines the steps involved in hepatic synthesis and secretion of VLDL.

In summary, under the stimulatory effect of oestrogen, VLDL and vitellogenin are synthesised by hepatocytes and released into plasma. On arriving in the developing oocyte they enter into the yolk via receptor-mediated endocytosis. Thereafter, within 20 minutes, a new arrangement takes place whereby apoB is cleaved into four smaller proteins, and vitellogenin into two phosvitins and two lipovitellines. The latter will form the core to which the particles will aggregate and form the 150μm yolk spheres. This process occurs over 7-10 days, until the follicle becomes 35-40mm in diameter when ovulation takes place.

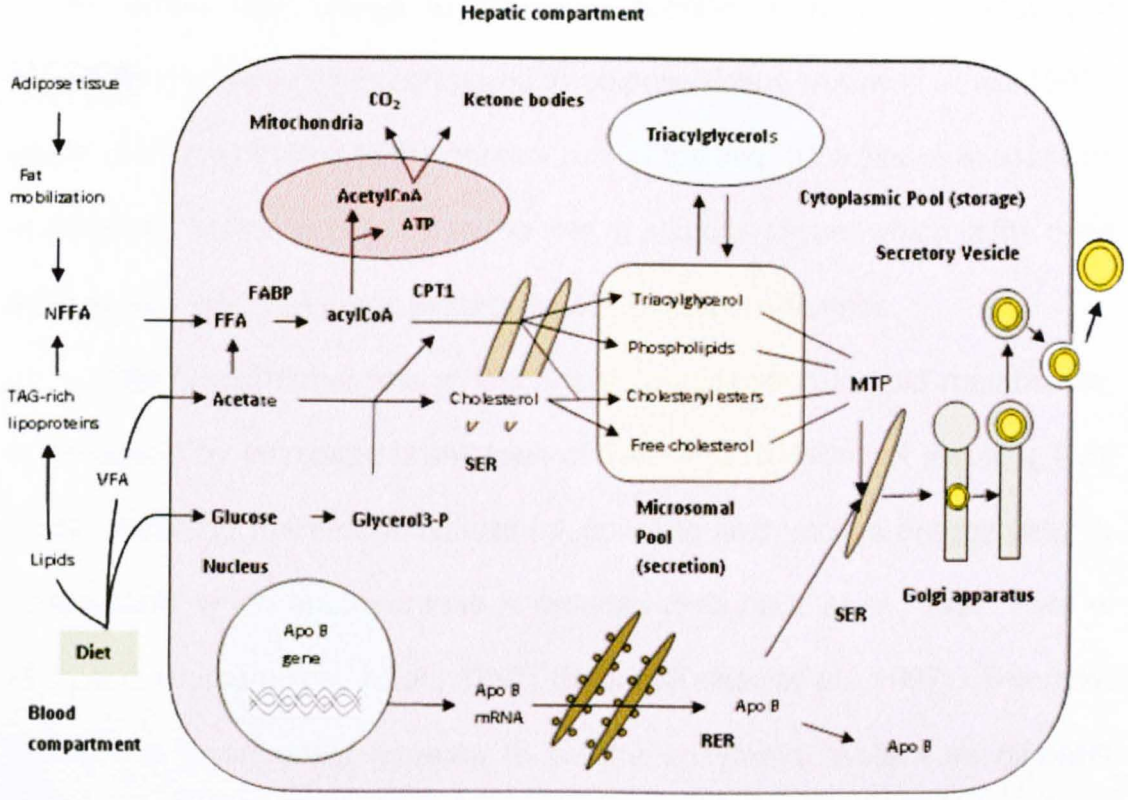


Figure 1.11 Schematic representation of VLDL synthesis and secretion in the liver (Gruffat *et al.*, 1996).

1.7.11 Role of Leptin in lipid metabolism

Leptin was first known in humans and rodents as a satiety hormone in reference to its role in controlling appetite which, in part, is achieved via decreasing the level of neuropeptide Y, an appetite stimulator (Stephens *et al.*, 1995). Leptin controls the appetite through many pathways (Beck *et al.*, 1993; 1995; Kristensen *et al.*, 1998; Luheshi *et al.*, 1999). Chicken leptin, first discovered and described by (Zhang *et al.*, 1994) consists of 145 amino acids with an unpaired cysteine residue at position 3 of the original cDNA. Leptin receptors are expressed in many tissues: hypothalamus, ovary, liver, intestine, kidney and pancreas (Touis *et al.*, 1998; Ohkubo *et al.*, 2000; Benomar *et al.*, 2000 cited by Taouis *et al.*, 2001; Kieffer & Habener, 2000).

Leptin action was shown to be tissue-specific, with more pronounced sensitivity of chicken liver compared to adipose tissue (Ashwell *et al.*, 1999), which can be attributed to the primary role of the liver as a site of lipogenesis in chickens. This is in contrast to the role of adipose tissue, which is the main storage site, and that might explain its lack of responsiveness.

The fundamental role of leptin is its participation in lipid metabolism, summarised by increased breakdown of TAG and oxidation of resulting fatty acids, switching from carbohydrate (glucose) to lipid use as energy-yielding components when energy intake is reduced (Frühbeck *et al.*, 1997; Hwa *et al.*, 1997; Shimabukuro *et al.*, 1997; Siegrist-Kaiser *et al.*, 1997). The main site of the leptin effect appears to be the enzymatic system as different studies showed the following enzymatic changes as a result of leptin administration:

- Inhibited expression of acetyl CoA carboxylase in adipocytes, responsible for the synthesis of fatty acids from carbohydrate (Bai *et al.*, 1996)
- Decreased expression of fatty acid synthase (Sarmiento *et al.*, 1997)
- Increased expression of hormone- sensitive lipase (Sarmiento *et al.*, 1997).

It was also suggested that leptin might cause adiopcyte apoptosis (Qian *et al.*, 1998a). Leptin expression is under hormonal and nutritional control (Taouis *et al.*, 2001; Macajova *et al.*, 2004). In relation to reproduction, oestrogen caused a decrease in leptin expression in both of liver and adipose tissue (Ashwell *et al.*, 1999; Karlsson *et al.*, 1997). These results were based *in vitro* research, thus more *in vivo* studies are needed to confirm these results under physiological conditions, where interactions of other regulatory factors may exist.

1.8 Reproductive cycle

Changes orchestrating the reproductive cycle can be summarized as follows. During the first 14 to 15 weeks of the female lifetime, the ovary is undeveloped and slow growing with the presence of follicles of only less than 3mm (diameter), which are the source of the slight increased levels of oestrogen present in the hen by 15 weeks of age. The presence of LH is not important for this increase to occur (Robinson & Etches, 1986). As birds receive the environmental cue (photostimulation) LH levels increase, leading to elevated levels of androgens and oestrogens, produced by theca cells, promoting appearance of the secondary characteristics and activation of the components of the reproductive network. One week prior to the first ovulation, as follicles grow, progesterone levels increase; oestrogen is believed to be essential to prime granulosa cells to produce progesterone (Wilson & Sharp, 1976; Etches & Duke, 1986). In response to this increase, GnRH is released from the hypothalamus into the hypothalamic-pituitary portal system leading to the preovulatory LH surge. For this to occur, both the hypothalamus and pituitary must first be primed by progesterone and oestrogens (Wilson & Sharp, 1976). Changes in hormone levels throughout the ovulatory cycle, defined as the interval between two ovulations that is classically 24-27 hours, are presented in Figure 1.12. This will continue up to 60 weeks of age when egg production decreases followed by cessation of lay. Thereafter, birds undergo moult, which is another criterion through which reproductive rejuvenation occurs in preparation for a new reproductive cycle and will be discussed subsequently.

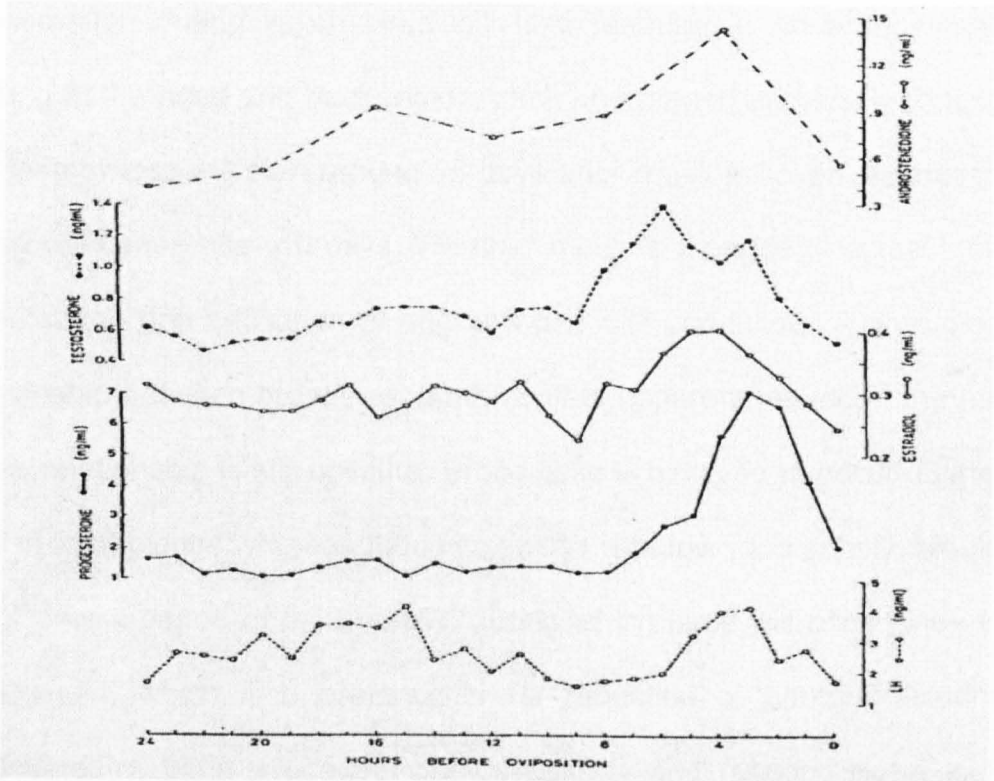


Figure 1.12 Plasma concentrations of: luteinizing hormone, progesterone, oestradiol, testosterone and androsteredione during the ovulatory cycle (Data from Etches and Cheng, 1981).

1.8.1 Termination of reproductive cycle

Consequential and naturally occurring events seem to be involved in the termination of egg production. Photoperiod is considered to have a prominent role in terminating as well as initiating the egg production cycle. Once a bird experiences increasing photoperiods, its hypothalamo-pituitary-gonadal axis is activated and, thereby, the reproductive cycle commences. Egg laying will continue until a day length is encountered when the bird will exhibit no further response to increasing photoperiod; this is photorefractoriness, during which the bird will stop laying eggs and intuitively tends to incubate its eggs – a condition known as broodiness. Another phenomenon initiated by long photoperiod is the moult. Moult is a

phenomenon during which birds will lose feathers in an orderly manner starting at the head and neck region, back and breast, and finally wings and tail; feather loss will start around 15 days after moult initiation, whereas egg laying will cease after 10 days. Whether moult is a cause or a result of the deterioration and cessation of egg laying is still undefined. A physiological relationship between broodiness and moult is apparent, although the nature of this relationship is still obscure. Broodiness is believed to occur during the time of food plenty, whereas moult coincides with low food supply. Moult will occur over a period of 8-12 weeks, during which birds will experience short days again, which is a prerequisite for reduction of photorefractoriness. Subsequently, birds will regain photosensitivity and respond again to long photoperiod allowing a new reproductive cycle to commence. Therefore, day length required to initiate photorefractoriness may be longer than that required for photostimulation (Etches, 1996; Rose, 1997; Leeson & Summers, 2000; Dawson, 1998a; Dawson *et al.*, 2001).

1.8.2 Reproductive aging

Egg laying rate varies throughout the egg production cycle, which consists of three periods. The first period is onset of reproductive activity; from the first oviposition, usually lasting over 1-2 weeks and possibly up to one month. Eggs laid during this period are characterised as mostly erratic, soft-shelled, and double-yolked, and there is irregular laying with large intervals between eggs. The second period is the normal laying period, when egg laying becomes regular and eggs are laid in clutches with one egg per day over a course of 18 - 30 days, followed by a pause day, in modern

strains. The third period is end of lay, during which a dramatic decline in reproductive activity occurs, ending in reproductive quiescence (Gilbert, 1969; Etches, 1990). A typical egg laying curve is outlined in Figure 1.13.

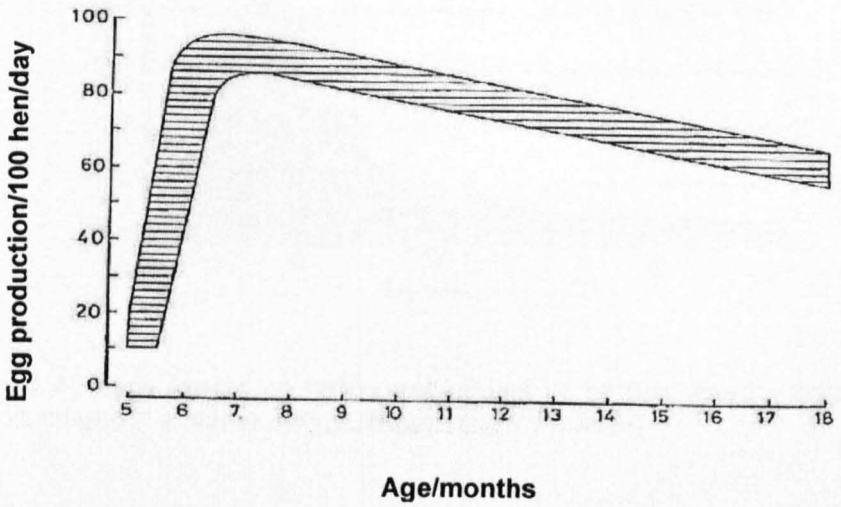


Figure 1.13 A typical egg production curve for a flock of laying hens expressed as the number of eggs laid per 100 hens per day. The normal range in production is indicated by the shaded area (Etches, 1990).

Many changes at the ovarian level characterise the end of lay period. Compared to younger birds, ovaries of older hens contain fewer small follicles, referring to all follicle types except LYF (Palmer & Bahr, 1992), with high incidence for the LYF to become atretic (Berry & Brake, 1985; Verheyen, 1987). Decreased rate of growth and maturation is a second feature of this period (Moudgal & Razdan, 1985; Johnson *et al.*, 1986; Palmer & Bahr, 1992), as pioneering studies postulated that a longer time is needed for yellow yolk material transportation to the growing follicle as the hen ages (Gilbert, 1971; Williams & Sharp, 1978). Taken together, these results might explain the shortened egg sequence, as few as one or two, and the increased interval between ovulations (Gilbert, 1969; Williams & Sharp,

1978b, Robinson *et al.*, 2003). Changes in egg sequence length throughout the egg production cycle are illustrated in Figure 1.14.

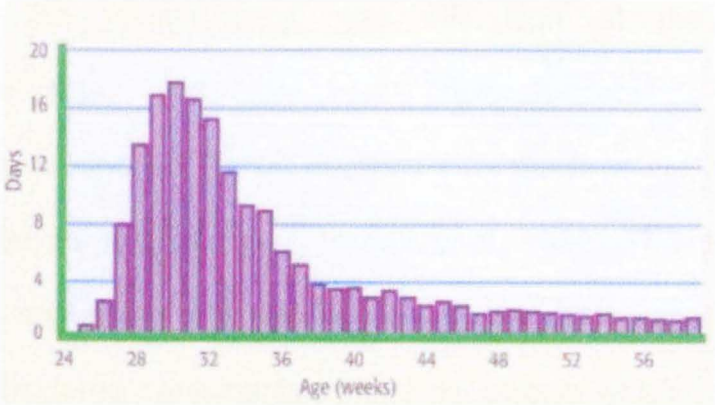


Figure 1.14 Average length of laying sequences of broiler breeder hens at 2-week intervals throughout a laying period (Robinson *et al.*, 2003)

1.8.3 Hormonal changes during end of lay period

By the end of reproduction cycle, many changes in the hypothalamic-pituitary-gonadal axis occur. As the hen ages, the hypothalamus becomes less sensitive both to increasing photoperiod (failure to convert the photon into neural signals), and to progesterone stimulation leading to decreased levels of GnRH, consequently reduced levels of both LH and FSH (Goldsmith & Nicholls, 1984; Dunn & Sharp, 1988; Etches, 1996). It has been suggested that the anterior pituitary loses its ability to secrete gonadotrophins in response to GnRH, because GnRH agonists, which regulate GnRH receptors, decrease (Kuenzel, 2003) and become less sensitive (Tanabe *et al.*, 1981). Decreased levels of FSH were suggested to be responsible for the lower follicular growth rate (Johnson *et al.*, 1986; Dunn & Sharp, 1988). FSH injection into old hens increased the number of small follicles (Waddington *et al.*, 1985), but not growth of the largest follicles in the hierarchy. The largest follicle, in turn, exhibits less sensitivity to LH as a hen ages (Johnson *et al.*,

1986), whereas decreased LH possibly leads to follicle atresia (Berry & Brake, 1985). It was presumed that GnRH release is inhibited while its content in the hypothalamus was still high at the beginning of photorefractoriness. Thereafter, as photorefractoriness evolves the hypothalamic content of GnRH declines (Contijoch *et al*, 1992; Reinert & Wilson, 1996a; Parry *et al.*, 1997; Meddle *et al.*, 1999). The reduced levels of LH and the suggested reduced sensitivity of the largest ovulatory follicle might be considered participants in follicular atresia which, in turn, leads to low levels of progesterone (Berry & Brake, 1985; Decuypere & Verheyen, 1986). This lowered level of progesterone seems to initiate feather papillae growth for rejuvenation (Rose, 1997). Steroids are also shown to decline by the end of the reproductive cycle (Hoshino *et al.*, 1988; Jacquet *et al.*, 1993; Joyner *et al.*, 1987; Ottinger *et al.*, 2002). This decline could be attributed to the decreased number of small follicles as hen ages. In addition, Dickerman *et al.* (1985) reported a reduced capability of theca cells to produce oestradiol in old hens.

1.8.4 Prolactin and Thyroxine

Prolactin concentration starts to rise slightly by the onset of egg laying and peaks concurrently with photorefractoriness (Ebling *et al.*, 1982; Dawson & Goldsmith, 1983). It was suggested that reproduction termination is brought about by prolactin acting as an inhibitory hormone. However, exogenous administration of prolactin did not cause photorefractoriness (Goldsmith, 1985). It was also found that, when transferring thyroidectomised birds from short to long photoperiods, prolactin level did not rise and

photorefractoriness did not occur (Dawson & Goldsmith, 1984). Another study showed that transferring birds from long photoperiods, but under the critical day length, to short days, prolactin levels did not increase, nor was photorefractoriness observed (Goldsmith & Nicholls, 1984c). However, with transfer to longer photoperiods, both a prolactin peak and onset of photorefractoriness were observed. The timing of high levels of circulating prolactin is linked to moult (Meier & MacGregor, 1972; Dawson & Goldsmith, 1983). Moult was prevented when prolactin release was suppressed (Dawson & Sharp, 1998). Thus, both prolactin increase and onset of photorefractoriness are needed for the onset of moult (Dawson & Sharp, 1998).

Thyroxine is another factor important for both initiation and continuance of photorefractoriness, and for termination of breeding (Goldsmith & Nicholls, 1984b; Wilson & Reinert, 1995a, b). Thyroxine is not a causative factor, but a permissive factor for switching off the reproductive system (Dawson, 1989a, b; Bentley, 1997). An increase in thyroxine level at the beginning of photostimulation was observed, suggesting a possible relationship between the thyroid gland and HPG axis (Reinert & Wilson, 1996). Subsequent studies confirmed the importance of thyroxine presence at one or two stages of converting photoperiodic information to GnRH-I neurons (Dawson *et al.*, 1986; Dawson, 1998b; Wilson & Reinert, 2000; Bentley *et al.*, 2000a).

Collectively, for the termination of egg-laying to occur, all these events are prerequisites with no single causative factor, but photoperiod is the key initiator of this series of events.

1.9 Role of calcium in the reproductive process

The presence of 2.3g and of calcium (Ca) in the shell and 25mg in the yolk indicates its valuable role in egg production. A total of 580g of Ca is secreted for shell formation when a hen produces 250-egg/yr. Therefore, if 0.50 of consumed Ca is kept for shell formation, the hen will release around 1.16kg of Ca through eggs per year (Etches, 1987). This underlines the participation of this element in the egg production process. Studies in the late 50s and 60s provided suggested the ability of the hen to estimate Ca loss and, surprisingly, to reactby reducing egg laying rate not by reducing shell deposition (Urist, 1959; Wood-Gush, 1963; Mehring, 1965). Diets of grossly inadequate Ca content might lead to cessation of egg laying (Gilbert, 1969; Etches, 1996; Johnson, 2000). Components involved in Ca transport are presented in Figure 1.15.

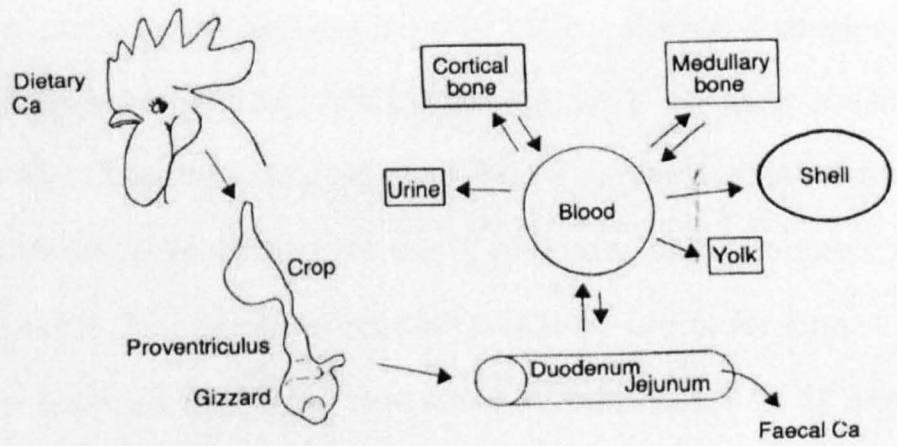


Figure 1.15 Components involved in calcium transport. Ca is ingested, stored in the crop, ground in the gizzard, and dissolved in the acidic environment of the proventriculus, and Ca^{+2} ions pass to the upper regions of the small intestine where they are transferred into blood. From the vascular system, Ca^{+2} can be utilized directly in shell formation or stored in either medullary or cortical bone. A small amount of Ca^{+2} is excreted in urine, and a significant quantity of Ca is excreted into faeces (Etches, 1996 modified from Etches, 1987).

Diet is the main Ca supplier during the day, and the medullary bone is the alternative supplier during the night when no feed is available. Approximately 100-200 mg/h of Ca is transferred for shell formation during darkness; as eggshells produced by hens fed diets containing labelled Ca contained layers of labelled, non-labelled and labelled Ca (Tyler, 1954).

1.9.1 Medullary bones in birds

The medullary bone is a strategy that is peculiar to birds to ensure adequate Ca supply for shell formation (as each egg contains around 0.10 of total body Ca; Nys, 1993; Whitehead, 2004) (Figure 1.16). "Physiological marrow ossification" was first reported by Kyes & Potter (1934) referring to the medullary bone, where long bones such as the femur and tibia are filled with spicules of bone, relating its formation to ovarian activity and the degree of ossification to the ovarian follicle size. Thus the medullary bone acts as a store of considerable amounts of labile Ca and medullary bones secure the structural skeleton (Miller, 1992). Medullary bone formation commences at the onset of sexual maturity (18wk of age); around 8-10 days prior to the first ovulation, under the combined effect of androgens and oestrogens (Common *et al.*, 1948). This period of time is considered critical for formation of this type of bone, as little or no new formation takes place at 37 and 43 wks (Hudson *et al.*, 1993).

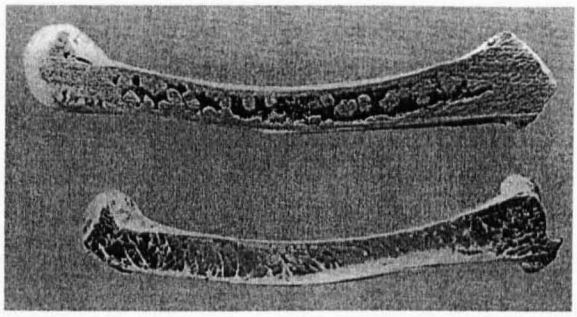


Figure 1.16 Structure of the medullary bone. A labile reservoir of Ca stored primarily in the long bones of sexually mature hens. The femur above illustrates the deposition of Ca phosphate as medullary bone in the hollow centre of the bone. The femur at the bottom, which does not contain medullary bone, is from a non-laying hen (from Taylor 1970; Etches, 1996).

1.9.2 Calcium level regulation

Two hormones control mobilization of medullary bone for shell formation: parathyroid hormone (PTH) primarily and calcitonin secondarily, in addition to the important role of the active metabolite of vitamin D; 1,25 dihydroxyvitamin D_3 (1,25 $(OH)_2D_3$). PTH exerts its effect on bone by instigating renal production of 1,25 $(OH)_2D_3$ which, in turn, regulates intestinal Ca absorption (Norman *et al.*, 1992); as both of them were found to increase during shell formation and they augment osteoclastic activity in the bone resulting in its breakdown to provide the required Ca (Taylor & Dacke, 1984; Hurwitz, 1989; Norman *et al.*, 1992).

Oestrogen plays a key role in regulating Ca level; high levels of oestrogen augment accumulation of Ca in the medullary bone. However, oestrogen decreases after ovulation and this seems to sensitize medullary bone, increasing osteoclastic activity in response to elevated levels of both of PTH and 1,25 $(OH)_2D_3$ (Castillo *et al.*, 1979; Van De Velde *et al.*, 1980; Johnson & Van Tienhoven, 1980; Etches & Cheng, 1981; Etches & Schoch,

1984). Oestrogen exerts its influence on gut, kidney and bones as a receptor regulator. It up-regulates 1,25 $(OH)_2D_3$ receptors in the gut, and PTH receptor in the kidney; which, in turn, activates the enzyme responsible for activation of 1,25 $(OH)_2D_3$ (Tanaka *et al.*, 1978; Forte *et al.*, 1983; Wu *et al.*, 1994). Another suggested role of oestrogen at the kidney level is that of improving Ca re-absorption in the tubule (Notelovitz, 1997). As oestrogen has been shown to up-regulate its own receptors, low levels of oestrogen lead to down-regulation of its receptors on bone, thus its antiresorptive effect on osteoclasts will decline, leading to their activation (Vaananen & Harkonen, 1996; Notelovitz, 1997). The presence of oestrogen receptor-α (OR α) in the avian duodenum has been confirmed, which might explain in part Ca uptake changes throughout the ovulatory cycle (Franzen *et al.*, 2002; Hansen *et al.*, 2003), which decreases as hens age.

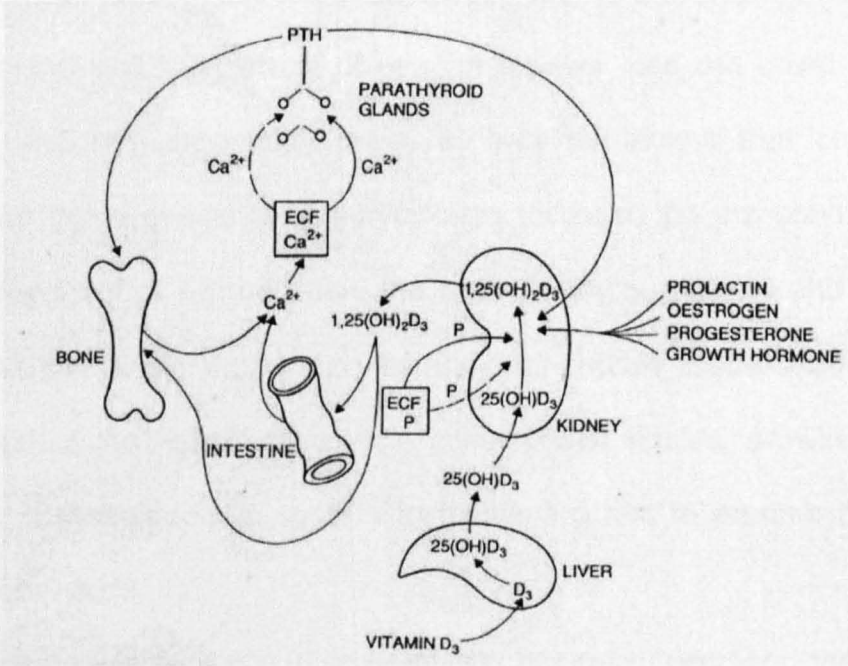


Figure1.17 Hormonal control of calcium mobilization (based on Etches, 1996 from Drezner & Harrelson, 1979), ECF: extracellular fluid.

The rise in the plasma Ca a few days prior to first ovulation occurs in birds laying non-calcified eggs and the bird will stop laying eggs when a Ca-deficient diet is given, although oestrogens are still secreted for 1-2 weeks. The suggested explanation for these observations is that vitellogenin, an important component of yolk material, is soluble in plasma when large amounts of Ca is available; as it binds large amounts of Ca, thus inadequate Ca will reduce the yolk material, thereby ovarian weight will decline followed by cessation of egg laying (Schjeide & Urist, 1956; Urist *et al.*, 1958).

Increased levels of oestradiol improve availability of Ca and its binding proteins, in addition to promoting osteoblastic activity. In contrast, lowered levels act as a signal to medullary bones; initiating osteoclastic activity to ensure the availability of Ca needed for calcification.

In summary, as egg yolks are the main material to sustain egg production, the aim of this literature survey was to shed light on the origin, development, and recruitment of ovarian follicles, and the onset of sexual maturity and egg production cycle, as well as factors that control and coordinate these events. The survey also focussed on the contribution of each component of reproduction, the hypothalamus, pituitary and ovary as well as the liver and medullary bones and their precise cooperation to initiate and terminate the reproductive cycle under photic signals, allowing birds to rest from the reproductive system to rejuvenate and to prepare for a new reproductive cycle.

These events were discussed as naturally-occurring phenomena. However commercial broiler breeders do not completely cease laying eggs, but the production deterioration they exhibit by the end of the reproductive

cycle is commercially uneconomic as forced moulting is illegal in the UK. Thus breeders are used for a single production cycle.

1.10 Genetic selection and egg production

Generations of genetic selection have culminated in broiler breeders that possess the heritable traits for rapid growth. However, these breeders are required to be good layers; in that they are expected to exhibit high rates of egg production, in addition to having the genetic potential which enables their progeny to grow faster.

Combining these two characteristics (reproductive and growth performance) has been difficult to achieve as the negative relationship between them is well documented (Maloney *et al.*, 1967; Jaap & Muir, 1968; Siegel & Dunnington, 1985). The body weight (BW) of broiler breeders is significantly higher as a direct result of the intensive genetic selection when compared to that of the original lines and egg-type hens (Chambers *et al.*, 1981; Hocking *et al.*, 1985; Hocking *et al.*, 1987; Hocking & McCormack, 1995; Hocking & Robertson, 2000;). Body weight differences between the egg-type and broiler breeder hens, as these two types are bred for distinct purposes, have been identified immediately post-hatching (Vieira & Moran, 1999; Zhao *et al.*, 2004) and even during embryonic development as the most recent studies have revealed (Sato *et al.*, 2006; Druyan, 2010). However, by the onset of reproductive activity, the differences between these two types become more crucial as body weight directly affects their reproductive performance. Differences in body weight between egg-type and broiler breeder hens are presented in Figure 1.18.

Egg production curves decline in both types of breeders late in the egg production cycle. In egg-laying strains egg production declines from greater than 0.90 to around 0.70-0.75 later in the egg production cycle; however, egg production rate in broiler breeders is about 0.55 as their egg production cycle is comparatively shorter (Johnson, 1993). Typical egg production curves of Shaver Single Comb White Leghorn (SCWL) hens and Indian River (IR) broiler breeder hens are presented in Figure 1.19. Females were photo-stimulated at 18 wk (SCWL) and 20 wk (IR) of age; SCWL hens were *ad-libitum* fed whereas broiler breeders were feed-restricted to typical industry levels. Females were maintained until 68 wk (SCWL) and 58 wk (IR) of age.

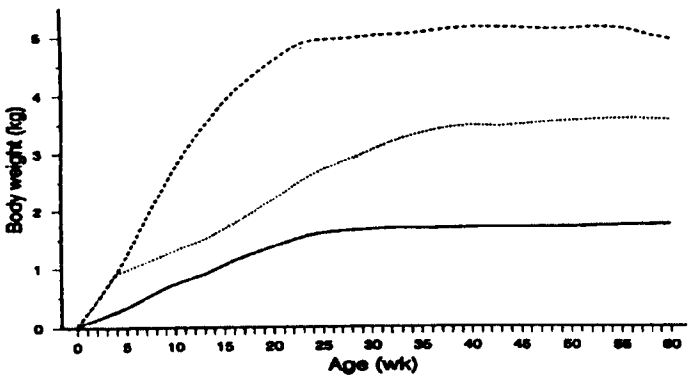


Figure 1.18 Typical body weight curves for full-fed Single Comb White Leghorn hens (—), Full-fed broiler breeders (– –), and broiler breeders that had been restricted to typical industry level (·····) from 0 to 60 wk of age (Robinson *et al.*, 1993 adapted from Summers & Leeson, 1985; Yu *et al.*, 1992a)

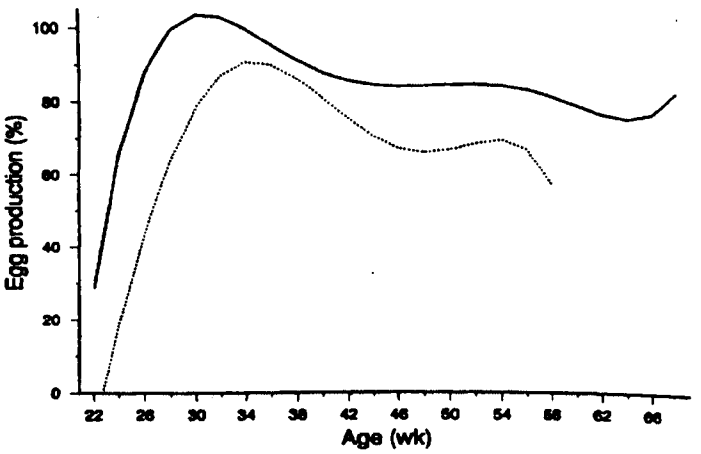


Figure 1.19 Profiles of percentage egg production for 160 (SCWL) hens (—) and 120 (IR) broiler breeders (·····) (Robinson *et al.*, 1993).

In summary, genetic selection for growth traits has resulted in increased appetite causing birds to become obese which, in turn, has impaired their reproductive performance. Studies undertaken in order to circumvent this problem have demonstrated feed restriction to be an efficient tool to solve this problem. In spite of having some negative outcomes for bird welfare, however, the positive effects of feed restriction on bird health, reproduction, and welfare were found to overweigh the negative impacts.

1.11 Aim of the study

The effect of genetic selection on body weight and consequently on reproductive performance has not only been observed between egg-laying and meat-type breeders as two distinct lines, but it was also found between developed broiler breeder strains and the original lines of meat-type chickens (Chambers *et al.*, 1981; Hocking *et al.*, 1985; Hocking *et al.*, 1987; Hocking & McCormack, 1995; Hocking & Robertson, 2000).

Genetic selection has continued for more growth traits such as breast-yield, at the same time these breeders are still required to have both rapid

growth and high rate of egg production to supply the generation of broiler chicks. However, balancing these requirements has been a critical issue, since selection for egg production is less heritable compared to selection for rapid growth. Thus, female breeders will allocate more energy for growth rather than egg production. An important issue could be raised with continuing genetic selection for more growth traits, which is whether this continued genetic selection has led to further deterioration in reproductive performance.

Ross 308 and 708 are two broiler breeder strains; the former represents a normal breast-yield broiler breeder strain, the latter represents a modern high breast-yield broiler breeder strain. Differences in breast meat weights between both strains at different ages are outlined in Figure 1.20 in a study undertaken by PD Hook (Breeders Ltd).

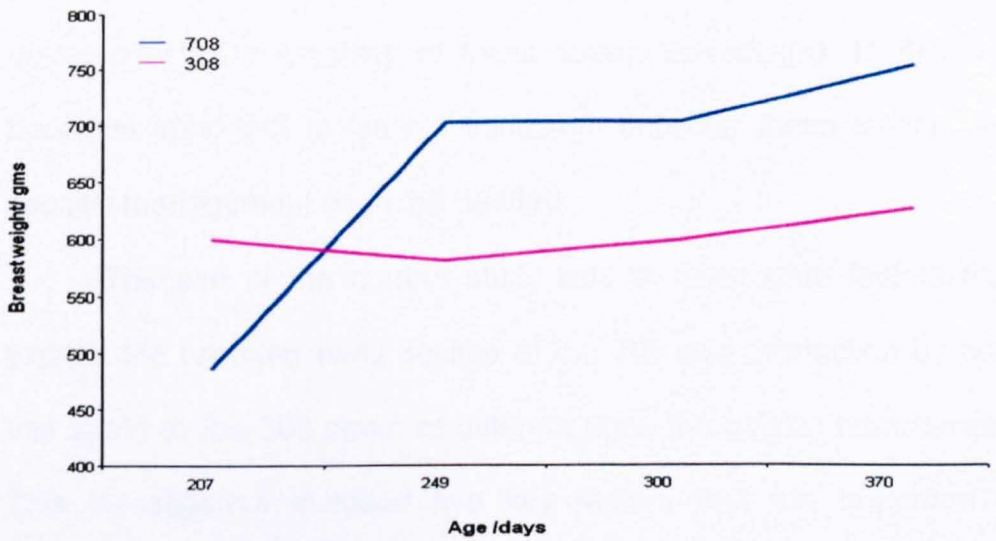


Figure 1.20 Breast weight as a proportion of carcass in 308 & 708 broiler breeders (PD Hook; Breeders Ltd).

Total egg production at 64 weeks was 180 for 308 and 172 for 708 according to Ross 308 and 708 Parent Stock Management Manuals. In practice, 708 breeders have been reported to exhibit rapid post-peak decline (personal communication; Daniel Dring, PD Hook). Under this situation, the reduced reproductive performance could be a result of the continued genetic selection for growth traits, or it might be due to individual differences regarding the balance between growth and egg production. Some females allocate more nutrients to egg production at the expense of growth, but this will not continue to the end of the reproduction cycle and at some point these birds will cease laying eggs. Another group where egg production hinders growth leads to more breaks in egg laying thus supporting growth (Renema *et al.*, 2006). Rapid post-peak decline could also be a management problem.

As the economical aim has always been to have breeders that perform efficiently in both growth and reproduction, a detailed understanding of reproductive physiology of these strains is required. In this context, it becomes important to identify variations between these strains, as strain-specific management might be needed.

The aim of the current study was to investigate factors that might explain the reported early decline of the 708 egg production by comparing this strain to the 308 strain at different ages throughout reproduction cycle. This investigation included five key factors that are important for egg production; first of these factors was ovarian follicle population; large yellow follicles (LYFs) as they will develop to eggs, small yellow follicles (SYFs) and large white follicles (LWFs) as they are the pool from which the LYFs are developed. As the main site of fatty acids synthesis in avian, the liver was the

second factor to be investigated in terms of fatty acid profiles, as it supplies the follicle with components that are important for follicle development. Body weight and fat content were found to impair reproductive performance; thus, they were studied as factors that reflect metabolic status and, consequently, variations in the metabolic hormones which have been found to act in concert with reproductive hormones and might underlie variations in reproductive performance. Calcium has also been shown to be an important element in the egg production process for calcification, mainly as a yolk material, to some extent, and for its important role for ovarian follicles proliferation and maturation as the recent studies have shown. Thus, calcium was investigated in the tibia bone to determine whether the reported egg production variation was associated with alteration in calcium metabolism. Hormone assay was the fifth factor to be investigated in the current study, including oestradiol which stimulates follicle growth, and some metabolic hormones that might affect reproduction such as leptin and thyroxine.

Chapter 2: Materials and Methods

2.1 Design of study

The current study was designed to compare two different broiler breeder strains reared on two different production units and on two different feeding and management regimes. A total of seventy broiler breeder females from Ross 308 and Ross 708 obtained from a commercial operation (PD Hook) at seven ages throughout the reproductive cycle starting at the onset of lay at 25-week-old to the end of the laying cycle at 55-weeks in five week intervals. Five female breeders per strain were collected at each age; ages and dates of collection are shown in Table 2.1.

Table 2.1 Information regarding birds collection; ages and dates

Age (wks)	308	708
25	29th January	19th February
30	5th March	26th March
35	2nd April	29th April
40	13th May	3rd June
45	17th June	8th July
50	15th July	12th August
55	19th August	16th September

2.2 Flock management

Broiler breeder females from both strains were fed standard diets according to PD Hook recommendations. Feeding regimes and nutrient specifications for rearing, pre-breeder and breeder periods are provided in Tables 2.2, 2.3 and 2.4. Broiler breeder females of both strains were photo-stimulated at 19-weeks old. Feeding allowances as well as lighting

programmes are provided in Appendix A (1, 2, and 3). All information regarding bird husbandry was provided by PD Hook.

Table 2.2 Feeding regimes for Ross 308 & 708 broiler breeder females from hatching to slaughter

Strain	308	708
Regime		
	Age/day	
S starter	0-14	0-14
starter	15-35	15-35
Grower 1	36-70	-
Grower 2	71-126	36-126
Pre-breeder	127-147	127-147
Breeder 1	148-245	148-273
Breeder 2	246-434	274-434

Table 2.3 Nutrient and energy specifications for diets fed to Ross 308 & 708 broiler breeder females during rearing period; S starter, starter, grower 1, and grower 2

	Units	S Starter	Starter	Grower 1	Grower 2
ME	MJ/kg	11.5	11.5	11	11
Protein	g/kg	200	175	155	145
Total lysine	g/kg	11	8.8	6.5	6
Total methionine+cystein	g/kg	8	7	5.8	5.5
Calcium	g/kg	11	11	10	10
Available Phosphorus	g/kg	4.5	4.4	3.8	3.7
Sodium	g/kg	1.5	1.5	1.5	1.4
Fish meal	g/kg	10	-	-	-
Choline	mg/kg	1500	1500	1400	1400

Table 2.4 Nutrient and energy specifications for diets fed to Ross 308 & 708 broiler breeder females during pre-breeder and breeder periods; breeder 1 and breeder 2

Diet	Pre-breeder	Breeder 1	Breeder 2
ME (MJ/kg)	11.40	11.45	11.45
Protein g/kg	145-150	145-150	140-145
Total lysine g/kg	6.5	6.8	6.5
Total methionine g/kg	3.1	3.3	3.1
Calcium g/kg	15-17	28-30	29-31
Available Phosphorus g/kg	3.5	3.4	3.3
Linoleic acid g/kg	12	12-13	11-12.5

2.3 Selection and handling of experiment birds

2.3.1 Birds selection and handling on arrival

Experiment breeder females were randomly selected, marked by different colours on tail or wing, and provided by two farms of PD Hook (Breeders Ltd). Ross 308 breeder females were supplied by Plump Bank Farm, Felixkirk, Thirsk, North Yorkshire, YO7 2EW, whereas Ross 708s were supplied by Hampsthwaite Farm, Glendale House, Hampsthwaite, Harrogate, North Yorkshire, HG3 2HL.

Birds were blood sampled by the company prior to collection, as part of routine metabolic monitoring regime, by hypodermic needle from vein under wing and transferred to tubes; 7.5cm long. Tubes were marked in different colours to match the bird from which they were obtained. Blood samples were transferred to Sutton Bonington in sealed tubes.

On arrival, birds were housed overnight at the Biological Resource Unit at Sutton Bonington to be processed early the next day. They were offered feed (provided by the supplier) and water *ad libitum*. Blood samples were put in a fridge at 4°C overnight and were centrifuged the following day using LP4 tubes (5ml, 75, 12mm, polystyrene) at 3000 rpm for 10 minutes, serum then was transferred into LP4 and stored at -20°C for hormone assay.

2.3.2 Birds processing

Birds were euthanized by Pentobarbital (Vétoquinol Ltd, UK) overdose via intravenous injection in the wing vein; amounts administered were calculated according to the manufacturing company recommendations as 0.7ml per kg body weight followed by cervical dislocation (both procedures

were conducted by trained and qualified personnel); dead weight was recorded.

Birds were cut open alongside the rib cage to the base of the wing, then the sternum was laid back to expose the organs in the abdominal cavity (Figure 2.1). All internal tracts were removed; liver and ovary of each bird were carefully removed and retained to be processed. Feet and shanks were removed at the tibiotarsal joint. One tibia bone was removed for Ca determination.

Ovarian follicles were detached, classified, and counted. Livers, after being weighed, and tibia bones were put in zip-lock bags, and kept in a polystyrene box on dry ice; thereafter, they were frozen at -20 ° C until processing, carcasses were then stored at -20 ° C.

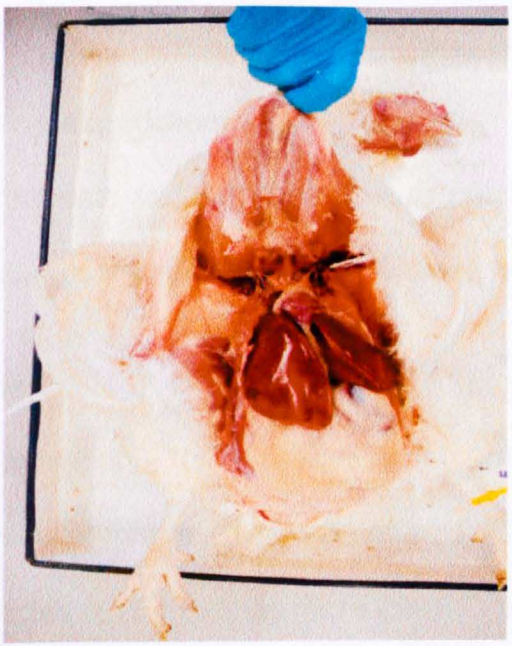


Figure 2.1 Bird dissection

2.3.3 Carcass processing

Carcasses were taken out of the freezer and allowed to partially thaw, then the head and crop were removed and cut into small cubes (around 5 cm^3) using an electrical band-saw. Using an electrical mincer (Hobart), carcasses were minced four times, mixed thoroughly, and representative samples of 200 g were taken and placed in pre-weighed containers (l litre rectangle plastic pots); the weight of the sample and container was recorded. Thereafter, samples were freeze-dried (Thermo Savant Super Modulyo freeze dryer, USA); samples were stored at –80°C for three hours prior to freeze drying. Once samples had consistent weight, they were ground using a mortar and pestle; for crude grinding and fine grinding an electrical grinder was used. Sub samples of approximately 30g were put in zip-lock bags and stored at -20 °C for total fat determination.

2.4 Measurements on experiment birds

All chemicals, reagents and equipment were purchased from Fisher Scientific, Leicestershire, UK and Sigma-Aldrich, Dorset, UK.

2.4.1 Ovarian follicles

After ovary separation, follicles were detached and classified according to the following categories (Robinson *et al.*, 2003)

- Large yellow follicles (LYF): follicles more than 10mm in diameter
- Small yellow follicles (SYF): follicles of 5-10mm in diameter
- Large white follicles (LWF): follicles of 1.2- 4.9mm in diameter

Follicles were then counted and their diameter was measured using a calliper (Figure 2.3).

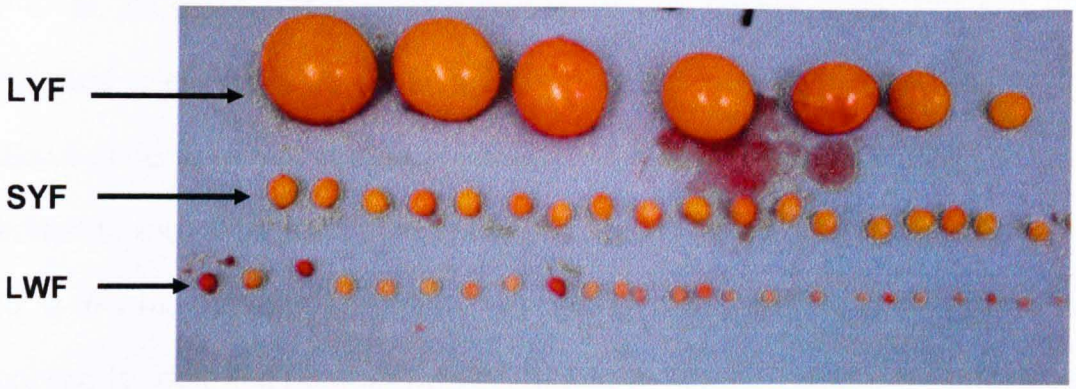


Figure 2.2 Follicle groups; LYF: large yellow follicles, SYF: small yellow follicles, LWF: large white follicles.

2.4.2 Liver processing

Livers were weighed immediately after separation. They were then freeze-dried; livers were placed in pre-weighed foil containers and the initial weights (liver + container) were recorded. Once weights became stable, they were ground using an electrical grinder, placed in zip-lock bags, and stored at -20°C for fatty acid analysis.

2.4.2.1 Lipid extraction and methylation

Lipid was extracted according to the method of Folch (Folch *et al.*, 1957) and methylated with 3N methanolic-HCl. About 50 mg freeze-dried liver was weighed into a 50ml Teflon-line, screw capped tube. A 1-ml of Nonadecanoate methyle ester (C19:0); a 100-mg of C19:0 methyl ester was dissolved in 100 ml hexane, was added as an internal standard and then vortexed for 5 minutes. A 5-ml of Folch solution (chloroform: methanol 2:1) was added and vortexed for 5 minutes to homogenize the sample. Samples then were left at 4°C for overnight. Thereafter, 3-ml of 0.9%NaCl, prepared

previously by dissolving 900 mg of Sodium Chloride in 100 ml of water, was added and vortexed for a few minutes, and then centrifuged for 10 minutes at 8°C at 3000rpm. After phase separation the bottom layer (≈3 ml) was carefully transferred into a 50 ml glass tube, dried in a block heater and blown under a gentle stream of nitrogen. A 3-ml of methanolic-HCl (3N) was added to the dried lipids, capped tightly, and then the mixture was vortexed for a few minutes and incubated in a heater block at 95°C for 1 hour. After cooling to room temperature, water (2ml) and hexane (2ml) were added and the mixture was vortexed for 2 minutes, and centrifuged for 10 minutes at 3000rpm at 8°C. An aliquot of the top layer was transferred to a 1.5ml vial, and then the sample was injected into the GCMS (Gas Chromatography Mass Spectrometry) for fatty acid detection. Samples were analyzed in duplicates.

Methylation method was modified from Wang *et al.* (2000) as nonadecanoate acid (C: 19) was used instead of tricosanoic acid (C: 23) because the latter acid did not completely dissolve in hexane.

2.4.2.2 Fatty acid methyl esters analysis and principle of GCMS

Fatty acid methyl esters (FAME) were detected using the GCMS (Agilent 6890N GC; Gas Chromatography system coupled with 5873N MSD; Mass Spectrometry Detector).

As the sample is injected into the GCMS it is carried by the carrier gas helium, the mobile phase, through a fused silica capillary column (Varian CP7489, 100 m length x 0.25 mm internal diameter x 0.20 µm film thickness), the stationary phase, held in an oven programmed to increase

the temperature gradually. The sample components held in the mobile phase interact with the stationary phase at different rates, and as the temperature increases compounds of different boiling points will elute from the column at different times; components with low boiling points will exit the column earlier than those of higher boiling points. As the components exit the GC column, they enter the mass spectrometer detector where they are bombarded with a stream of electrons causing the breakdown of these components into fragments which are actually charged ions with a certain mass and considered as a fingerprint used to identify the component by its molecular weight.

Practically, components are shown as a graph with many peaks, each peak represents a different component with different retention time (RT); the time from sample injection (time zero) to the time of elution when the peak reaches the maximum height (the height of the peak shows the concentration of this component). Using a compound of known retention time makes identification of unknown components possible; this compound is referred to as the standard. In the current study a standard of 37- fatty acid methyl esters was used; where 1ml of the standard FAME (fatty acid methyl esters) was made up to 10ml with dechloromethane CH2CH2 (Carsinogine, organic solvent) and a sample of 1.5 ml was injected into the GCMS to get the chromatogram of the fatty acids which was used to identify the fatty acids in the liver samples.

Figure 2.2 and Table 2.5 show the 37 peaks of the fatty acid methyl esters of the standard used to identify liver fatty acids.

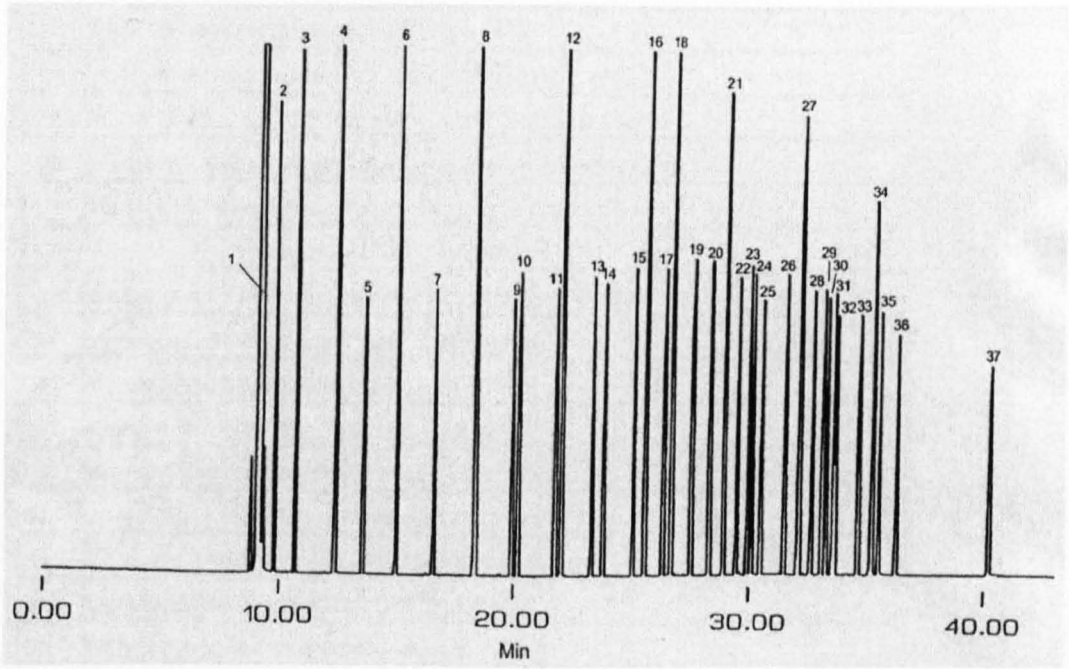


Figure 2.3 Chromatogram of the 37 fatty acid methyl esters standard mixture

Table 2.5 Components of fatty acid methyl esters standard mixture

1.	Butyric Acid Methyl Ester (C4:0) at 4 wt %
2.	Caproic Acid Methyl Ester (C6:0) at 4 wt %
3.	Caprylic Acid Methyl Ester (C8:0) at 4 wt %
4.	Capric Acid Methyl Ester (C10:0) at 4 wt %
5.	Undecanoic Acid Methyl Ester (C11:0) at 2 wt %
6.	Lauric Acid Methyl Ester (C12:0) at 4 wt %
7.	Tridecanoic Acid Methyl Ester (C13:0) at 2 wt %
8.	Myristic Acid Methyl Ester (C14:0) at 4 wt %
9.	Myristoleic Acid Methyl Ester (C14:1) at 2 wt %
10.	Pentadecanoic Acid Methyl Ester (C15:0) at 2 wt %
11.	cis-10-Pentadecenoic Acid Methyl Ester (C15:1) at 2 wt %
12.	Palmitic Acid Methyl Ester (C16:0) at 6 wt %
13.	Palmitoleic Acid Methyl Ester (C16:1) at 2 wt %
14.	Heptadecanoic Acid Methyl Ester (C17:0) at 2 wt %
15.	cis-10-Heptadecenoic Acid Methyl Ester (C17:1) at 2 wt %
16.	Stearic Acid Methyl Ester (C18:0) at 4 wt %
17.	Oleic Acid Methyl Ester (C18:1n9c) at 4 wt %
18.	Elaidic Acid Methyl Ester (C18:1n9t) at 2 wt %
19.	Linoleic Acid Methyl Ester (C18:2n6c) at 2 wt %
20.	Linolelaidic Acid Methyl Ester (C18:2n6t) at 2 wt %
21.	γ-Linolenic Acid Methyl Ester (C18:3n6) at 2 wt %
22.	α-Linolenic Acid Methyl Ester (C18:3n3) at 2 wt %

23.	Arachidic Acid Methyl Ester (C20:0) at 4 wt %
24.	cis-11-Eicosenoic Acid Methyl Ester (C20:1n9) at 2 wt %
25.	cis-11,14-Eicosadienoic Acid Methyl Ester (C20:2) at 2 wt %
26.	cis-8,11,14-Eicosatrienoic Acid Methyl Ester (C20:3n6) at 2 wt %
27.	cis-11,14,17-Eicosatrienoic Acid Methyl Ester (C20:3n3) at 2 wt %
28.	Arachidonic Acid Methyl Ester (C20:4n6) at 2 wt %
29.	cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester (C20:5n3) at 2 wt %
30.	Heneicosanoic Acid Methyl Ester (C21:0) at 2 wt %
31.	Behenic Acid Methyl Ester (C22:0) at 4 wt %
32.	Erucic Acid Methyl Ester (C22:1n9) at 2 wt %
33.	cis-13,16-Docosadienoic Acid Methyl Ester (C22:2) at 2 wt %
34.	cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester (C22:6n3) at 2 wt %
35.	Tricosanoic Acid Methyl Ester (C23:0) at 2 wt %
36.	Lignoceric Acid Methyl Ester (C24:0) at 4 wt %
37.	Nervonic Acid Methyl Ester (C24:1n9) at

2.4.2.3 Quantification of fatty acids

The content of each fatty acid; expressed as g/kg, was calculated according to Golay *et al.* (2006) as the following

$$FAi = \frac{m_0 . A_i . R_i . S_i (FA) . 100}{A_0 . m}$$

m_0: mass of the IS added to the sample solution, in mg

A_i: peak area of the $FAME_i$ in the sample

R_i: response factor is the mean of two injections of the calibration standard solution for each $FAME_i$ present in the calibration standard solution and is calculated relative to the internal standard 19:0

$$R_i = \frac{m'_i . A'_0}{m'_0 . A'_i}$$

m'_i: %mass of the $FAME_i$ in the calibration standard solution

A'_0: peak area of IS in the calibration standard solution chromatogram

m_0': % mass of IS in the calibration standard solution

A'_i: peak area of the $FAME_i$ in the calibration standard solution

S_i (FA): stoichiometric factor used to express correctly results as fatty acids and is the ratio between the methyl esters and fatty acids molecular masses (e.g. the oleic acid (C 18:1) methyl ester molecular weight is 296 and the oleic acid molecular weight is 282. Thus, S_i = 296/282 = 1.05).

A_0: peak area of the IS

m: mass of the sample (ground liver) in mg

2.4.3 Total carcass fat determination

Carcass fat content was determined at Food Science, School of Biosciences, University of Nottingham for samples of the first two ages of both strains; 25 and 30 weeks of age. The remaining samples were analysed at the International Laboratory Services (ILS), Shardlow, Derbyshire.

Carcass fat content was determined according to the Association of official analytical chemists (AOAC) official methods 950.46 (forced air oven drying) (AOAC, 2007) and 960.39 (rapid Soxhlet extraction) (AOAC, 2007) (Food Science), and according to the AOAC official method 2008.06 microwave drying and nuclear magnetic resonance (AOAC, 2007) (in ILS). Samples were packed in a polystyrene box within a cardboard box, and ice packs were put inside to maintain a temporary low temperature during transport to the ILS. Carcass samples were analyzed in duplicate.

2.4.3.1 Principle of rapid Soxhlet extraction

In this method the extraction solvent, petroleum ether, was placed in a flask, heated until it boils and evaporates and passes through the sample, placed in the extractor to the condenser where it is cooled and condensed to small drops and drips back to the sample. When the chamber containing the sample is loaded with the solvent, this solvent containing the dissolved fat will automatically be emptied down to the distillation flask. Steps for fat extraction are in Appendix B.

2.4.3.2 Principle of microwave drying and nuclear magnetic resonance (NMR)

In this method the sample is dried in the microwave to constant weight to, and then the dried sample will be placed into the NMR, which sends a pulse of radio frequency (RF) energy through the sample, which will be absorbed by hydrogen protons present in the different constituents of the sample, generating a signal known as free induction delay (FID). This signal from protein and carbohydrate decays very rapidly; however, for fat it decays relatively slowly. The system software will then measure the FID to determine the amount of fat protons in the sample and the fat content then is displayed on the screen as a percentage (g/100g). Samples preparation is detailed in Appendix C.

It has been shown that fat determination using microwave drying and NMR provided equivalent results to those of oven dried and Soxhlet ether extracted. Keeton *et al.* (2003) compared the fat content of different meat products using the official methods of forced air oven drying and rapid Soxhlet extraction with the results derived from using the official method of

microwave drying and NMR. Both methods were shown to give equivalent results for the different meat products studied; fat content for chicken meat was 72.4 g/kg with the former method and 72.9 g/kg using the latter method.

2.4.4 Tibia processing

2.4.4.1 Ashing and preparation for calcium determination

Bones were removed from the freezer and allowed to partially thaw, they were then thoroughly cleaned and all external tissues were removed using a knife and a single edge razor. Thereafter, they were weighed, dried at 105°C for 24 hrs and weights were recorded. Samples were returned to the drying oven overnight and re-weighed. Bones were then cut into small sections using a hacksaw; weight of the pieces was recorded so that bone losses incurred during the sawing process could be estimated. Samples were returned to the drying oven overnight and re-weighed. Drying was continued until a constant weight was achieved. When completely dry, the bone pieces were ashed at 600°C for 12 hrs, allowed to cool and then weight was recorded. Samples were then ground into a fine powder, in the crucibles, using a pestle.

Concentrated hydrochloric acid (2 ml) was added to approximately 100mg of ash weighed into a 30ml universal, and left overnight until completely dissolve giving a clear solution. The solution was then transferred into a 100 ml volumetric flask, washed with distilled water and added to the volumetric flask; distilled water was added until 100ml mark.

Concentrated hydrochloric acid (2 ml) was added into a separate 100ml volumetric flask and made up with distilled water until 100ml, this was

used as a blank sample. Procedures followed were according to (Pointillart *et al.*, 1995).

Subsamples of 1ml were added into a 30ml tubes and diluted by adding 9ml distilled water and injected into the Inductively Coupled Plasma Mass Spectrometry (ICP-MS; Thermo-Fisher Scientific X- Series^{II}). Samples were analyzed in triplicate; results were expressed as g/kg.

2.4.4.2 Principle of the inductively coupled plasma mass spectrometry (ICPMS)

Once the sample is injected into the ICP-MS, it mixes with the argon gas to form an aerosol, and passes through the extreme high temperature argon plasma. Consequently it will be dried and converted to gaseous atoms which absorb energy and release an electron. This will result in the formation of positively singly charged ions which will enter the mass spectrometry where they will be separated according to their mass-to-charge ratio. As they reach the detector they will be counted as an electrical signal which, in turn, will be translated into a concentration using Plasmalab software (version 2.5.4; Thermo-Fisher Scientific).The latter step was undertaken in the School of Biology, University of Nottingham, where the samples were sent for Ca determination.

2.4.5 Hormone assays

Following unforeseen deterioration of blood samples, hormone assays were not undertaken. After removing serum tubes from the freezer, they were found coagulated with brownish colour. Some samples still contained some serum, so it was decided to undertake leptin assay; however, leptin levels were found to be much less than the normal levels according to the

literature, thus results were not considered, and no more hormone assays were undertaken.

2.5 Birds excluded

Some birds were excluded from the study because they had undeveloped ovaries; information regarding these birds is provided in Table 2.6.

Table 2.6 Birds excluded from the study; ages, strain, and number

Age/wks	Strain	Number
30	308	1
35	708	3
50	308	1

2.6 Statistical model

All statistical analyses were carried out using Genstat v.12 (VSN International, UK). A polynomial ANOVA was employed to analyze liver fatty acids content, body weight, carcass fat, and calcium content in tibia bone, because this study was undertaken at sequential ages and this design allows testing for a linear (Lin) or a quadratic (Quad) trend of the studied variables over the time and it also allows detecting more complex responses; deviations (Dev). However, generalized linear model Poisson distribution with log link function was used to analyze ovarian follicle counts as they do not follow normal distribution. The main effects were age and strain with five replicates in each strain; as each bird was considered as a replicate. Statements of statistical significance were based on $P \leq 0.05$. R^2 values were calculated using Microsoft excel 2007. Standard error was expressed

as SE for follicles number, whereas standard error of differences was expressed as SED for the rest of the parameters studied.

Chapter 3: Ovarian Follicular Dynamics in Ross 308 and 708 Breeders at Different Ages

3.1 Introduction

Ovarian follicle development commences as early as day ten of embryonic development with the number of primary oocytes that the hen will ever have being established by the time of hatching. Although thousands of these primary oocytes exist, only, 250-500 of them are ovulated throughout the female breeder lifespan (detailed in Chapter 1; 1.6).

Generations of genetic selection of broiler breeder strains for growth traits has impaired the balance between growth and reproductive fitness, causing reduced reproductive capabilities of the broiler-parent stock (Maloney *et al.*, 1967; Jaap & Muir, 1968). This has been well addressed when comparing growth and reproductive performance of egg-laying and broiler breeder hens (detailed in Chapter 1; 1.10). The continued genetic selection of the current, high breast-yield broiler breeder strains demonstrate continued increases in the growth potential. However, whether or not this has additional negative impacts on egg production is still to be determined.

Ross 708 represents one of these modern strains and has been reported to have lower egg production compared to the more traditional 308s (personal communication; Daniel Dring, PD Hook). As the chicken's ovary contains follicles, the main material that will develop into an egg, at different stages of development that are detachable and countable, this makes the ovary the site where the reproductive status can be reliably measured.

Thereby, the ovary was the first of the reproductive components investigated and counting follicles was one of the aims of the current study.

Follicles were classified as described in Chapter 2 (2.4.1). Data were analyzed as described in Chapter 2 (2.6).

3.2 Results

3.2.1 Flock data

Egg production profiles for the flock, from which the experimental birds were sampled, were provided by PD Hook and are presented in Figure 3.1. These data show that 708 broiler breeder females developed for high breast-yield exhibited less egg production over the early weeks of their reproductive cycle compared to the conventional breast-yield 308. However, no difference was detected between 35- and 55-weeks old.

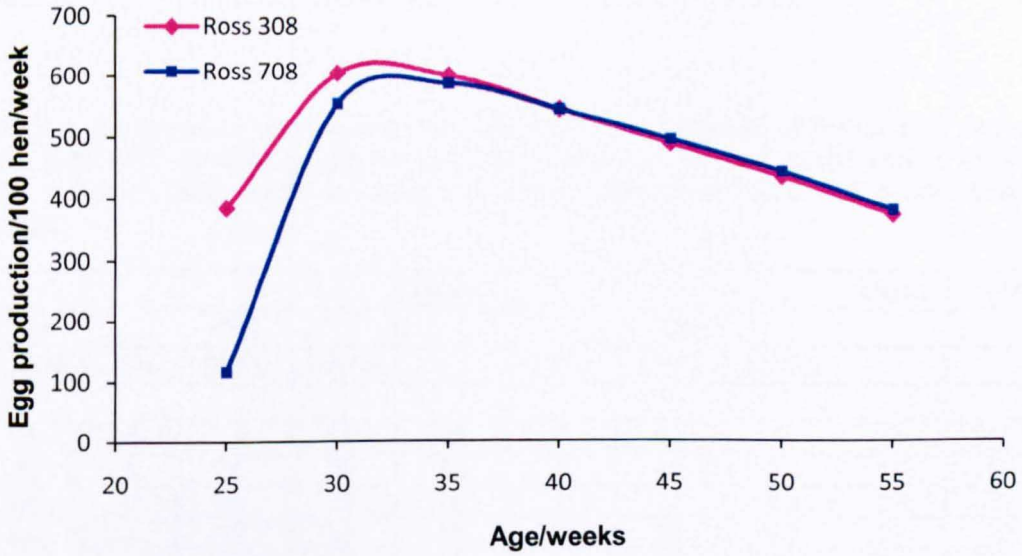


Figure 3.1 Egg production profiles expressed as numer/100 hen/week for Ross 308 & 708 broiler breeder females (PD Hook).

3.2.2 Experiment data

3.2.2.1 Effect of age and strain on the ovarian follicles number

The effect of age and strain on the number of follicles in different categories, and the statistical analysis of these effects are presented in Tables 3.1, 3.2.

Age had a significant effect on the number of the different follicle types studied. Both large yellow follicles ($P<0.001$) and small yellow follicles ($P=0.015$) decreased with advanced age, whereas large white follicle numbers increased significantly ($P<0.001$).

Females from both strains did not differ significantly in their large and small yellow follicle number. However, ovaries of the 708s contained significantly ($P=0.005$) more large white follicles.

There was no age by strain interaction in the number of large yellow follicles and large white follicles. However, there was a trend for an interaction ($P=0.075$) in the number of small yellow follicles.

Table 3.1 Influence of age (weeks) and strain on the number of follicles of different types (LYFs=10 mm; large yellow follicles, SYFs = 5-10 mm; small yellow follicles, LWFs = 5 mm; large white follicles) of ovaries from Ross 308 & 708 broiler breeder females.

				Mean				Mean	Total
Strain	308				708				
Follicle	LYFs	SYFs	LWFs		LYFs	SYFs	LWFs		
Age									
25	8	15	13	12	7	13	11	11	11
30	8	26	28	20	8	13	24	15	17
35	6	23	26	18	7	22	40	23	20
40	6	22	26	18	6	21	40	22	20
45	6	17	37	20	7	22	45	24	22
50	5	18	53	25	5	18	72	32	29
55	6	17	44	22	6	12	76	31	27
Mean	6	20	32	19	7	17	44	23	21

Table 3.2 Analysis of Generalized Linear Model showing the effect of age and strain on the number of the large yellow follicles (LYFs), small yellow follicles (SYFs), and large white follicles (LWFs) in Ross 308 & 708 broiler breeder females.

	LYFs		SYFs		LWFs	
Factor	SE	P	SE	P	SE	P
Age	0.4	<0.001	2.0	0.015	4.9	<0.001
Strain		0.332		0.134		0.005
Age*Strain		0.904		0.075		0.192

3.2.3 Effect of age and strain on the largest yellow follicle diameter

The statistical analysis of the effect of age and strain on the diameter of the largest yellow follicle is presented in Tables 3.3 and 3.4

Age had a significant effect (P<0.001) on the diameter of the largest yellow follicle; as there was a linear increase (P<0.001) and there was also a quadratic (P<0.001) effect of age (a decrease).

There was a trend for a smaller diameter in the 708s (P=0.054). No age by strain interaction was found.

Table 3.3 Influence of age (weeks) and strain on the largest yellow follicle diameter (mm) of Ross 308 &708 broiler breeder females.

			Mean
Strain	308	708	
Age			
25	32	30	31
30	35	33	34
35	35	36	36
40	37	35	36
45	37	37	37
50	38	33	36
55	36	36	36
Mean	36	34	35

Table 3.4 Analysis of Variance showing age and strain effects on the largest yellow follicle diameter of Ross 308 & 708 broiler breeder females.

Factor	SED	P
Age	1.2	<0.001
		<0.001 (Lin)
		<0.001 (Quad)
		0.789 (Dev)
Strain	0.6	0.054
Age*Strain	1.7	0.259
		0.970 (Lin)
		0.721 (Quad)
		0.113 (Dev)

3.3 Discussion

Chickens are classified under the short lifespan theme and they exhibit reproductive aging (Holmes *et al.*, 2003), which is represented as a decreased egg production. In broiler breeder strains, birds reach the peak of egg production 6-8 weeks after the onset of lay which is sustained between 30 and 45-50 weeks of age followed by a conspicuous decline at around 50 weeks of age (Ottinger, 1992).

Studies have been undertaken to identify physiological changes in the hypothalamic-pituitary-ovarian axis that underlie reproductive failure with age. The endocrinological changes at the level of the hypothalamic-piyuitary-ovarian axis are detailed in Chapter 1 (1.8.3).

At the level of the ovary, the large yellow follicles (LYFs) have been given specific attention as they reflect, to large extent, egg production rate. Statistical analysis of the current study has shown a significant decrease in the number of this type of follicles with advanced age. There have been different views that explain egg production deterioration coincidental with advanced age in relation to the number, growth, or functional changes that

occur in this type of follicle. Some researchers found that follicular growth becomes slower in aged hens (Wilson & Cunningham, 1984; Palmer & Bahr, 1992). Williams and Sharp (1978) suggested a change in the pattern of yolk packaging into the follicles; yolk material being packaged in a fewer number of follicles which grow to a larger size before ovulation. In the case of hens having complete hierarchies and yet showing a remarkable decrease in egg production towards the end of their first reproductive cycle, the internal ovulation and the failure to oviposit the yolk into hard-shelled eggs were suggested to be responsible for this failure (Wood-Gush & Gilbert, 1970). Lillpers and Wilhelmson (1993) summarised their results as the following; as the hen ages the time needed for follicle maturation and egg formation becomes longer resulting in long oviposition intervals and consequently shorter egg sequences. This might also result in more than a one day pause, in addition to the increased incidence of missing eggs with advanced age. They also suggested that the older hen becomes unable to balance the energy required for follicular growth and egg formation. Lacassagne (1960) found that between 44 and 78 weeks of age the time taken for a follicle to reach an ovulatable state increased by 22 hours. It is well documented that thecal tissues of the largest ovulatory follicle lose the capacity to produce androgens from the progesterone produced by granulosa cells 12 hours before ovulation, which contributes to the elevated level of progesterone required for ovulation (Robinson & Etches, 1986). Lebedeva *et al.* (2010) found the largest ovulatory follicle in old hens is still capable of producing androgens and that lowers the level of progesterone needed for ovulation to

occur. Large yellow follicles also become more vulnerable to atresia with advanced age (Johnson *et al.*, 1980; Berry & Brake, 1985; Verheyen, 1987).

With regard to the small yellow follicles (SYFs) and large white follicles (LWFs), Palmer & Bahr (1992) found that in comparison to younger hens, ovaries of older hens contained fewer of these two types of follicle. However, there is little information in the literature regarding the number of these types of follicle at different ages. In relation to LWFs, Hoshino *et al.* (1988) reported that a decreased level of oestradiol was concomitant with cessation of egg production. Ottinger (1996) stated that failure in ovarian function during the process of aging is accompanied with a decreased number of small follicles and decreased production of steroid. In contrast, (Senior & Furr, 1975; Gilbert & Wells, 1984; Joyner *et al.*, 1987; Herremans *et al.* 1988) reported that the decline in the oestradiol level at the end of the reproductive cycle was not significant, and they explained that as the white follicles produce about 87% of oestrogens, this may indicate that there is no decrease in the number of white follicles. However, none of these studies have investigated the dynamics of white follicles throughout the egg production cycle. The increased number of LWFs towards the end of the first reproductive cycle, found in the current study, could be one of the ovarian preparations for the new reproduction cycle. As many changes have been reported in the other reproductive components during the transition period between two reproductive cycles; rejuvenation in the oviduct, pituitary gland and bones were reported (Heryanto *et al.*, 1997a; 1997b; Yoshimura *et al.*, 1997; Chowdhury & Yshimura, 2002; Whitehead, 2004).

In the current study, the number of SYFs was investigated as it is the pool from where LYFs are recruited, and LWFs form the pool for SYFs. However, no relationship was found between the numbers of these different follicle types; no relationship between LYFs and SYF (R^2= 0.03) or SYFs and LWFs (R^2= 0.01). Consequently, it can be inferred that the number of SYFs does not have any effect on the number of LYFs. The basis on which a follicle is recruited into the LYFs hierarchy is still to be determined and follicle size does not have any role in this recruitment (Woods & Johnson, 2005), according to the observations of the current study, the number the SYFs could also be excluded.

Ovaries from both strains of breeders did not differ in their content of LYFs and SYFs, but the LWFs did differ. However, the significant difference found in the LWFs did not result in any difference in the other follicle types. Most research has concentrated on LYFs and SYFs, and, most importantly, on LYFs as they are the main material that will develop into an egg. In contrast, the LWFs have not been given much attention in investigations throughout the reproduction cycle. When researchers mention this type of follicle, it is usually as a minor point. However, the large numbers of this type of follicle seems to be common with advanced age. Their numbers seem to vary between strains, as will be discussed later. In a study undertaken by Waddington and Hocking (1993) to investigate LYFs and white follicle distribution in the anterior and posterior halves of the ovaries of either *ad-libitum* or restricted fed broiler breeder females at 47-week-old, the number of white follicles was 72.8 for *ad-libitum* and 75.6 for restricted breeders. In another study, conducted by Ebeid *et al.* (2008) on 50-week-old Mandarah

breeder females (Egyptian cross), in order to investigate the effect of catecholamine injection on promoting follicular development and ovulation, the number of white follicles was 188. Many points could be applied here; the dominance of the LWFs as the hen advanced in age was demonstrated in both strains, in agreement with the studies mentioned, regardless of the numbers, which could be said to be one of reproductive aging signs. In the two studies mentioned, white follicles were classified into size groups; 1.4-2.4 mm and 2.4-5.0 mm diameter (Waddington & Hocking, 1993), 1-3 mm and 3-5 mm diameter (Ebeid *et al.*, 2008) with dominance of the smaller size group in both studies; this was found also in the current study. However, no separate count was done. Large white follicles are the source for oestrogens that stimulate production of gonadotrophin releasing hormones from the hypothalamus and gonadotrophic hormones from the pituitary gland which are important for follicular development and ovulation. However, there should be a level at which the hypothalamus responds to oestrogen stimulation and when that level is exceeded a negative feed-back occurs rather than stimulation which might, in turn, impair follicular development and consequently egg production. The reduced levels of these hormones by the end of the reproductive cycle are well documented as factors contributing to termination of the reproduction cycle (Goldsmith & Nicholls, 1984; Dunn & Sharp, 1988; Etches, 1996). These researchers attributed these changes to the reduced sensitivity of the hypothalamus to increased photoperiod and to progesterone stimulation which, in turn, leads to reduced levels of GnRH, consequently reduced levels of both FSH and LH from the pituitary. In controlled poultry houses, the photoperiod is the same from the onset of lay

to the end of reproduction; 14 hours in the current study, yet all these changes take place. Thus, there must be additional factors that contribute to termination of egg production. Changes in steroid levels have also been studied; however, with contradictory results. Some studies reported a decline in steroid production by the end of lay due to a reduced number of small follicles (Hoshino *et al.*, 1988; Jacquet *et al.*, 1993; Ottinger *et al.*, 2002). Other studies found no significant change in steroid levels between young and old hens (Senior & Furr, 1975; Gilbert & Wells, 1984; Joyner *et al.*, 1987; Herremans *et al.* 1988). Variations in results could be attributed to the age at which these studies were undertaken. For example, Joyner *et al.* (1987) undertook their study on young layers at 30- to 40-weeks old, and old layers at 150- to 200-weeks old which, in turn, were grouped into old hens still in lay or non-layers. Moreover, according to the studies of (Waddington & Hocking, 1993; Ebeid *et al.*, 2008), it seems that the number of large white follicles differs from one strain to another.

This might be in agreement with the early egg production decline reported by PD Hook regarding the 708s and also with some morphological differences of the ovary from both strains that will be mentioned later in this discussion. As it was only possible to investigate five birds from each strain at each time point, the assumption must be made that these birds were representative of the whole flock at that time point. Had it been possible to study a larger number of birds at each time point, better representation of the flock would be achieved. Reproductive hormones, such as oestrogen, LH, and FSH, studied at the different ages might have explained the effect of LWFs.

Although no difference was found between strains in LYF number, the 708 breeders have been reported by PD Hook to exhibit lower egg production. In the current study, however, there was no difference in egg production profiles of the two flocks from which the experimental birds were collected (Figure 3.1). However, having the same number of the LYFs does not necessarily mean that the breeders will lay the same number of eggs. It is not certain that all of these follicles would develop into an egg at the same rate, as follicles might take different times to become mature and ovulatable. This can be seen in the current study at 25-week-old and, to some extent, at some time points between 25- and 30-week-old as there was a difference in egg production between 308 and 708, in spite of the lack of difference in number of LYFs.

Some morphological anomalies were noticed on the ovaries of 708s; some ovaries contained complete hierarchies which represent the normal situation, others contained different sizes of LYFs and some had atretic LYF (Figure 3.2). The abnormal observations can be indicators of shorter sequences, leading to more empty days and lower total egg production. It is also possible in the case of the two atretic LYFs that the breeder stops laying eggs; such cases have not been noticed in the 308 breeders.

Regarding the largest follicle size change with age, follicle size increased with advanced ages. Many studies have investigated follicle size at different ages; an early study showed that follicle size increased by 29% during the first 8 weeks of production (Warren & Conard, 1939). Williams and Sharp (1978) found that the largest follicle ovulates at a smaller size in young hens (26-week-old) in comparison to that of the old ones (52- and 113-weeks

of age). Other studies reported the increased size of the largest ovulatory follicle after 60-70 week of age (Zakaria *et al.*, 1983; Johnson *et al.*, 1986; Joyner *et al.*, 1987). These researchers suggested two explanations for the increased size of the largest ovulatory follicle at advanced ages; the first is the prolonged time taken for the largest follicle to reach ovulatable status, which allows more time for yolk material to accumulate in the follicle; the second is the increased rate at which yolk material is transported into the developing follicle, this suggestion stemmed from the finding that the rapid growth period did not increase with advanced age (Gilbert, 1971a; Williams & Sharp, 1978). Results of the current study are in agreement, to large extent, with these findings; the largest yellow follicle diameter increased linearly to peak at around mid-lay and decreased thereafter (quadratic; $P<0.001$). This change will be discussed in relation to liver fatty acids in the general discussion chapter.

In conclusion, age significantly affected ovary content of follicle types in both 308 and 708 breeders. Both LYFs and SYFs decreased significantly with advanced age, whereas LWFs increased. No significant difference in LYFs and SYFs was detected between strains; however, 708 ovaries contained significantly more LWFs. Although the number of LYFs did not differ between strains, some morphological differences in this type of follicle were observed which could contribute to shortening egg-laying sequence and therefore reducing the total egg production of 708 breeders in comparison to their 308 counterparts. However, this speculation cannot be confirmed before thorough investigation of egg-laying sequence length in larger samples over several generations.

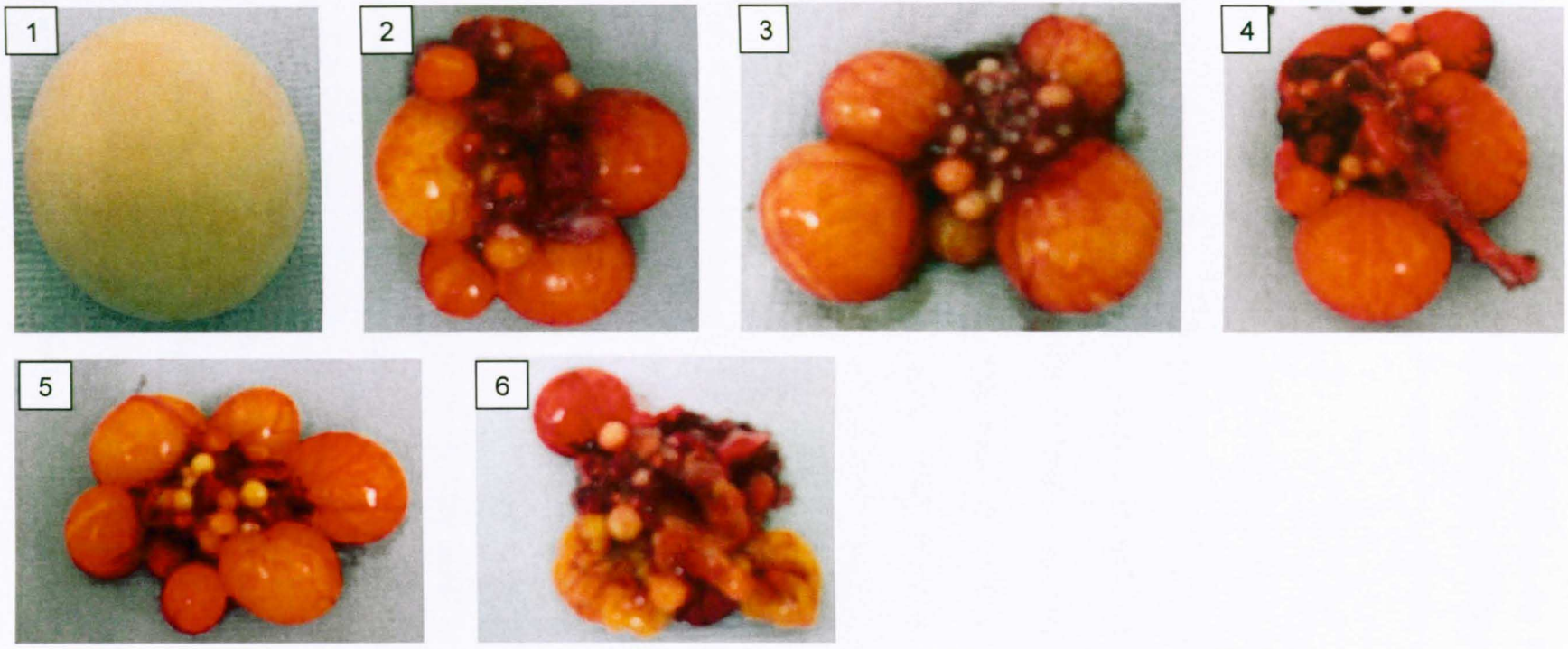


Figure 3.2 Photos showing different situations of the ovaries of 308 and 708 broiler breeder females.
1: Found in the body cavity of a 40-week-old 708 broiler breeder female, 2: The ovary of a-45-week-old 308 broiler breeder female
3: The ovary of a-40-week-old 708 broiler breeder female, 4: The ovary of a-45-week-old 708 broiler breeder female showing different sizes of the follicles,
5: The ovary of a-45-week-old 708 broiler breeder female showing a complete hierarchy, 6: The ovary of a-50-week-old 708 broiler breeder female showing two atretic large yellow follicles

Chapter 4: Liver Fatty Acid Profiles in Ross 308 & 708 Broiler Breeder Females at Different Ages

4.1 Introduction

Adipose tissue and the liver are the two sites responsible for lipogenesis; however the predominant site differs between species. In ruminants, pigs and dogs adipose tissue is considered the principal site of lipogenesis (O'Hea & Leveille 1969b; Baldner *et al.*, 1985), whereas in birds, humans and fish the liver is the primary site where this process takes place (O'Hea & Leveille 1969a; Shrago *et al.*, 1971; Lin *et al.*, 1977). In rats and rabbits both adipose tissue and liver are of importance (Ballard *et al.*, 1969; Leung & Bauman, 1976).

The difference in lipogenic capability between adipose tissue and the liver in birds might be attributed to the expression of an important transcription factor, sterol regulatory element binding protein-1 (ADD1/SREBP-1), which controls expression of a number of enzyme genes that are involved in lipogenesis such as fatty acid synthase, acetyl CoA carboxylase, ATP citrate lyase, steroyl CoA desaturase and malic enzyme (Shimano *et al.*, 1999).

Triacylglycerols (TAG), the main product of hepatic lipogenesis, are transported from the site of synthesis to the growing oocyte in the form of very low density lipoprotein (VLDL) particles. Transferring TAG in this form is a highly efficient process where they are transported in a combination of two specific apoproteins conferring a unique structure to the VLDL particle. This

structure ensures effective transportation of the TAG-rich VLDL particle as well as facilitating TAG entry to the developing oocyte (detailed in Chapter 1, 1.7.10).

As a hen lays approximately 275 eggs during her first reproductive cycle, she is producing around 5.5kg of yolk of which 3kg is solid material with a high proportion of lipoprotein. This reflects the precise role of the liver in yolk formation thereby sustaining egg production (Griffin *et al.*, 1984). Although it was shown that VLDL particles are assembled within the endoplasmic reticulum of some cells of the kidney in roosters, these particles do not possess the adequate structure that allow them to access the oocyte. Thus, these VLDL particles supply tissues express lipoprotein lipase (LPL) such as cardiac muscle, skeletal muscle and adipose tissue (Blue *et al.*, 1980; Walzem *et al.*, 1999). Thereby, hepatic VLDL is the yolk-targeted VLDL.

Since the embryo develops in a shelled egg, the maternal phase is represented in the formation and packaging the required nutrients in the yolk. Of the yolk constituents TAG, accounting for 0.65 of yolk lipids, supplies 189kJ of a total of ≈ 357kJ contained in a 60-g hen egg (Kuksis, 1992) as the energy is considered a limiting factor for embryonic growth and development. The production of these specific particles of VLDL starts during the laying period, as studies have revealed that VLDL from the plasma of laying hens or oestrogen-treated chicks is different from that of immature hens or roosters (Gornall & Kucsis, 1973; Kudzma *et al.*, 1979). It was also shown that the plasma of the laying-hen has high levels of VLDL particles. The fatty

acid composition of TAG in plasma VLDL particles was shown to be identical to that of yolk (Christie & Moore, 1972).

Taken together, the liver is of considerable importance in the formation of the different lipid components which are assembled in the form of VLDL particles that have a specific structure to ensure the intact TAG transport to the growing oocyte. Thus, the liver plays a crucial role in reproduction via its participation in oocyte growth, hence sustaining a daily production of an egg by the laying hen.

In general, fatty acids consist of even numbers of carbon atoms (2-36) in straight chain with a carboxyl group at one extremity. The most common fatty acids in animal tissues are those containing 14-22 carbon atoms. Fatty acids are classified as:

- Saturated fatty acids: fatty acids with straight chain, the most abundant saturated fatty acids in animal tissues are myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0).
- Monoenoic fatty acids: straight-chain fatty acids with even numbers of carbon atoms (10 to more than 30) containing one cis or trans double bond, which can be found at different positions in relation to the carboxyl group. Oleic acid (C18:1(n-9), with n referring to the number of carbon atoms between the terminal methyl group and the first double bond) is the most abundant monoenoic fatty acid in animal tissues.
- Polyunsaturated fatty acids (PUFA): are those fatty acids containing from 2 to 6 double bonds. Linoleic (C18:2(n-6)) and α-linolenic (C18:3(n-3)) acids are essential PUFA as they cannot be synthesized in animal tissues and they

are major precursors of the long chain PUFA by desaturation and chain elongation (Figure 4.1).

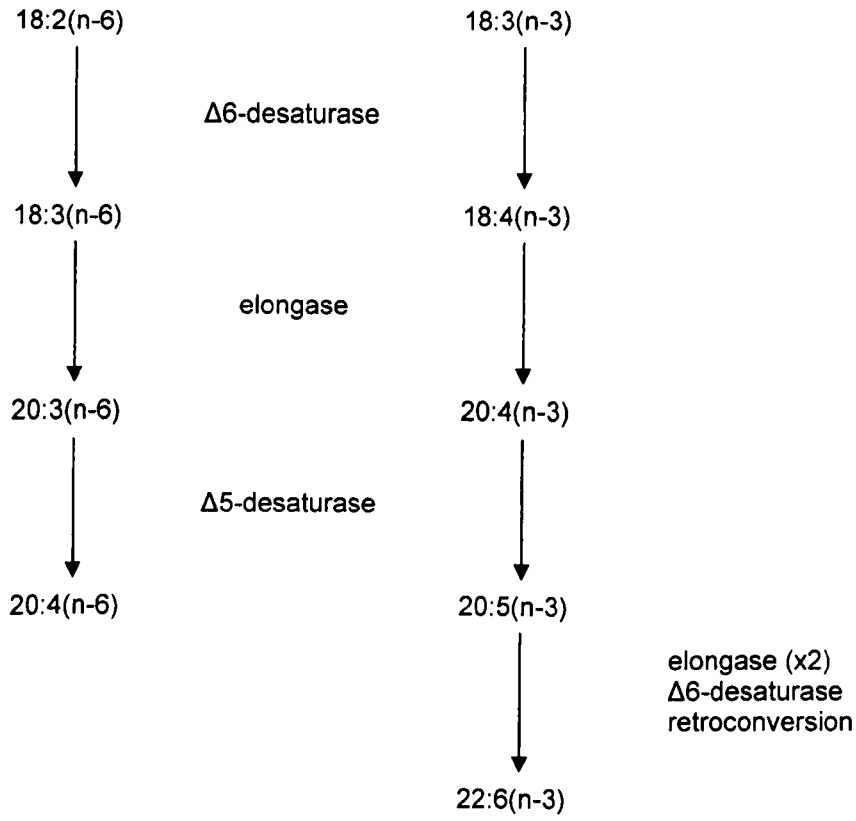


Figure 4.1 Biosynthesis of long chain polyunsaturated fatty acids by chain elongation and desaturation of linoleic and linolenic acids (Christie, 2003).
18:2(n-6) is linoleic acid, 18:3(n-3) is α-linolenic, 20:3(n-6) is dihomo α-linolenic, 20:4(n-6) is arachidonic acid, 20:5(n-3) is eicosapentaenoic acid (EPA) and 22:6(n-3) is docosahexaenoic acid (DHA).

As fatty acids are the major components of VLDL, in addition to their important physiological role in living organisms, and since the liver in chickens is the main site for fatty acids synthesis, liver fatty acid profiles were investigated in order to determine whether a fundamental difference in lipid metabolism between 308 and 708 does exist which, in turn, might affect their reproductive performance.

Liver fatty acids were analyzed and their amount was calculated as described in Chapter 2 (2.4.2). Fatty acids investigated are presented in Table 4.1.

Results were analysed as described in Chapter 2 (2.6).

Table 4.1 Trivial names and shorthand designation for the fatty acids investigated in the liver of Ross 308 & 708 broiler breeder female.

Trivial name	Shorthand designation
Palmitic acid	C16:0
Palmitoleic acid	C16:1
Stearic acid	C18:0
Oleic acid	C18:1(n-9)
Linoleic acid	C18:2(n-6)
γ-Linolenic acid	C18:3(n-6)
α-Linolenic	C18:3(n-3)
Dihomo γ-Linolenic acid	C20:3(n-6)
Arachidonic acid	C20:4(n-6)
Eicosapentaenoic EPA	C20:5(n-3)
Docosapentaenoic DPA	C22:5
Docosahexaenoic DHA	C22:6

4.2 Results

The effect of age and strain on the liver content of the different fatty acids and the statistical analysis of these effects are presented in Tables 4.2 and 4.3.

4.2.1 Effect of age on liver fatty acid profiles

Age had a significant effect on lipid metabolism. Most fatty acid levels were significantly affected by age except arachidonic and EPA (P values were 0.322 and 0.113, respectively) although there was a trend for a quadratic effect on the former acid (P=0.099) and for a linear effect on the latter (P=0.060). For all remaining fatty acids age effect was linear (P<0.001, however, P value was 0.004 for γ-linolenic acid). In addition to the linear

effect of age there was also a quadratic effect with some fatty acids; stearic (P=0.048), α-linolenic (P=0.023), and dihomo γ-linolenic (P=0.028) with a trend for a quadratic effect with DPA (P= 0.053). Additionally, statistical analysis showed that there were deviations from linear with some fatty acids; palmitic (P= 0.002), palmitoleic and oleic (P= 0.005), linoleic (P<0.001), and DHA (P= 0.018), whereas there was a strong trend for deviations from linear (P= 0.054) with γ-linolenic acid. However, there were deviations from quadratic with other fatty acids; stearic (P= 0.014), α-linolenic (P= 0.010). There was a trend for deviations from quadratic (P= 0.078) with dihomo α-linolenic acid and also a strong trend for quadratic effect (P= 0.053) and for deviation from quadratic (P= 0.051) with DPA.

4.2.2 Effect of strain on liver fatty acid profiles

There was no significant difference between 308 and 708 in the content of saturated fatty acids; palmitic and stearic acids; although there was a trend for 708 to have higher content of palmitic acid (P values were 0.071 and 0.354, respectively). Palmitoleic fatty acid did not differ between strains (P=0.185) whereas this difference was significant for oleic acid (P=0.022).

There was a strong trend for lower content of linoleic acid in 708 (P=0.056). However, the contents of both γ-linolenic and dihomo γ-linolenic were significantly less in 708 (P values were 0.005 and <0.001, respectively), but no significant difference was found in arachidonc acid content (P=0.869). With regard to (n-3) fatty acids, α-linolenic acid was significantly lower in 708 (P=0.005), the same response was observed for DPA and DHA (P<0.001 for both fatty acids), however EPA did not differ significantly (P=0.334).

4.2.3 Effect of age and strain interaction on liver fatty acid profiles

Age and strain interaction had no effect on some fatty acids; palmitoleic (P=0.302), dihomo γ-linolenic (P=0.592), and EPA (P=0.208). There was a quadratic effect of the interaction with saturated fatty acids; palmitic acid (P=0.028) in addition to a strong trend for deviations from quadratic responses (P=0.052), and stearic acid (P<0.001), there was also significant deviations from quadratic responses with stearic acid (P= 0.003).

Age and strain interaction had a significant quadratic effect on linoleic acid (P= 0.002); although this fatty acid is being added to the diet, its content differed between strains, notably, at 40-week-old. There was also a significant quadratic effect on the content of γ-linolenic acid (P=0.035). There was also a linear and a quadratic effect of the interaction on arachidonic acid content (P values were 0.043 and 0.01, respectively). Taking into consideration that linoleic acid is the precursor of γ-linolenic and arachidonic acids; this might explain the difference in their contents between 308 and 708 seen at some time points.

There was a quadratic effect of age and strain interaction on the content of α-linolenic acid (P=0.047) as well as deviations from quadratic (P=0.007). DPA and DHA both were affected by the interaction; the interaction effect was linear and quadratic for both fatty acids (P values for DPA were 0.037 and 0.004, respectively, and for DHA were <0.001 and 0.015, respectively).

Table 4.2 Influence of age (weeks) and strain on the liver fatty acids content (g/kg) of Ross 308 & 708 broiler breeder females.

								Mean								Mean	Total
Strain	308								708								
Age	25	30	35	40	45	50	55		25	30	35	40	45	50	55		
Fatty acid																	
C16:0	36	37	43	37	27	26	27	33	41	52	44	26	28	30	36	37	35
C16:1	6	7	7	6	4	5	5	6	6	9	8	5	5	3	6	6	6
C18:0	14	14	14	14	11	9	10	12	15	18	13	9	9	12	13	13	12
C18:1n9	52	56	63	51	38	35	36	47	59	78	65	40	40	51	54	55	51
C18:2n6	19	21	21	22	13	11	13	17	18	22	19	15	11	12	15	16	17
C18:3n6	0.10	0.17	0.16	0.14	0.11	0.07	0.08	0.12	0.09	0.14	0.09	0.05	0.06	0.07	0.08	0.08	0.10
C18:3n3	0.74	0.74	0.67	0.98	0.57	0.40	0.40	0.64	0.60	0.71	0.72	0.51	0.41	0.34	0.45	0.53	0.59
C20:3n6	0.27	0.34	0.27	0.34	0.26	0.16	0.18	0.26	0.20	0.29	0.21	0.20	0.15	0.15	0.12	0.19	0.23
C20:4n6	3	3	3	2	3	2	2	3	3	3	2	2	2	3	3	3	3
C20:5	0.08	0.13	0.37	0.07	0.05	0.03	0.02	0.11	0.13	0.08	0.08	0.05	0.05	0.06	0.05	0.07	0.10
C22:5	0.25	0.36	0.27	0.35	0.28	0.08	0.07	0.24	0.14	0.24	0.10	0.09	0.11	0.10	0.11	0.13	0.19
C22:6	2.01	2.20	1.75	1.83	0.66	0.42	0.37	1.32	1.32	1.37	0.90	0.65	0.52	0.75	0.56	0.87	1,12
Total	133	142	154	135	99	89	94	121	144	184	153	100	97	112	128	131	127

Table 4.3 Analysis of Variance; showing the effect of age and strain on the liver fatty acids content of Ross 308 & 708 broiler breeder females.

Factor	Age					Strain		Age * Strain				
Fatty acid												
	SED	P	P (Lin)	P (Quad)	P (Dev)	SED	P	SED	P	P (Lin)	P (Quad)	P (Dev)
C16:0	3.6	<0.001	<0.001	0.593	0.002	1.9	0.071	5.0	0.03	0.679	0.028	0.052
C16:1	0.9	<0.001	<0.001	0.796	0.005	0.5	0.185	1.2	0.302	0.345	0.948	0.18
C18:0	0.9	<0.001	<0.001	0.048	0.014	0.5	0.354	1.3	<0.001	0.687	<0.001	0.003
C18:1n9	6.3	<0.001	<0.001	0.833	0.005	3.3	0.022	8.8	0.146	0.598	0.065	0.202
C18:2n6	1.1	<0.001	<0.001	0.141	<0.001	0.6	0.056	1.6	0.008	0.182	0.002	0.109
C18:3n6	0.023	0.007	0.004	0.284	0.054	0.012	0.005	0.032	0.311	0.407	0.035	0.745
C18:3n3	0.069	<0.001	<0.001	0.023	0.01	0.037	0.005	0.098	0.007	0.582	0.047	0.007
C20:3n6	0.033	<0.001	<0.001	0.028	0.078	0.018	<0.001	0.047	0.592	0.821	0.315	0.474
C20:4n6	0.25	0.322	0.381	0.099	0.473	0.13	0.869	0.35	0.03	0.043	0.01	0.412
C20:5	0.069	0.113	0.06	0.414	0.179	0.037	0.334	0.097	0.208	0.437	0.156	0.207
C22:5	0.045	<0.001	<0.001	0.053	0.051	0.024	<0.001	0.064	0.016	0.037	0.004	0.45
C22:6	0.158	<0.001	<0.001	0.945	0.018	0.085	<0.001	0.224	<0.001	<0.001	0.015	0.041

4.3 Discussion

Fatty acid profiles have been regularly investigated in yolks of broiler breeders as they are the major energetic source for embryonic development and growth. Many factors were found to contribute to the changes of yolk fatty acid profiles; genetics (Horbanczuk *et al.*, 1999), season (Pandey *et al.*, 1989), ration (leskanish & Noble, 1997) and age (Latour *et al.*, 1998; Nielsen, 1998; Burnham *et al.*, 2001; Yilmaz-Dikmen & Sahan, 2009). The liver is the main site where linked, sequential, enzyme-catalyzed reactions take place and fatty acids are synthesized and packaged to be transported to the yolk. However, there is little information in the literature regarding liver fatty acid profiles.

Results of the current study showed that concentrations of most liver fatty acid were affected by hen age, indicating a change in lipid metabolism as the hen advanced in the laying cycle.

Studies on rat hepatocytes (Schmucker & Jones, 1975; Schmucker, 1976) reported a decreased density of the smooth endoplasmic reticulum, which suggested a change in the hepatic function in older rats. Also, responsiveness of the hepatocyte to lipogenic agonists was found to decrease as the chickens advanced in age (Harris *et al.*, 1988). Therefore, the reduced fatty acid contents of the liver might be attributed to deterioration in hepatic capacity to synthesize fatty acids.

Liver contents of some fatty acids varied significantly between strains; oleic acid was higher in 708 (P=0.022) compared to 308, also there was a tendency for 708 to have more palmitic acid (P= 0.071). This might suggest different activities of enzymes responsible for fatty acids synthesis between

308 and 708. Acetyl CoA carboxylase, fatty acid synthase, malic enzyme and glucose-6-phosphate are the four key enzymes that regulate lipogenesis, in addition to desaturase and elongase enzymes responsible for PUFA synthesis. The activity of these enzymes is controlled by hormones, as it has been shown that hormones exert their effect via regulatory regions existing on the gene coding the enzyme protein synthesis; each hormone has its regulatory region of a specific sequence on the said gene. Thus alteration of enzymatic activity occurred via altering the rate of its synthesis, and hence its concentration (Goodridge *et al.*, 1991). Hodnett *et al.* (1996) identified a region on the malic enzyme gene (106 kb length) in the 5 kb 5`-flanking DNA that contained regulatory element of specific sequence and have been found to respond to triiodothyronine (T_3) once the latter binds to specific receptors on the malic enzyme gene. Salati *et al.* (1991) found that incubating a chick-embryo hepatocyte with T_3 for 24 hours resulted in a 40-fold more mRNA for malic enzyme when compared to its amount without T_3 addition. The combination of T_3 and insulin did not show any change in the maximal amount of the mRNA reached when T_3 was added alone. However, the latter role was found to amplify the former effect; as around 0.80 of the increase in the mRNA amount caused by the addition of T_3 alone was reached within a few hours rather than 24 hours. As hormones are the signals that communicate the physiological status to the different parts of the body including the liver where they contribute to the regulation of the hepatocyte activity, this might suggest physiological differences existing between both strains which might alter enzymes activity.

The lower concentrations of both γ-linolenic (P=0.005) and dihomo γ-linolenic (P<0.001) acids found in 708 livers may be attributed to the availability of linoleic acid, the main substrate for both. Although there was only a tendency for the 708s to have lower content of linoleic acid, there was a significant age by strain interaction at 40-week-old which coincided with big differences in the levels of these fatty acids between both strains. In the current study, linoleic acid was added to the diet of both breeders at two levels according to age; 308 received 12.0-13.0g linoleic acid /kg until 35-week-old and 11.0-12.5 g/kg thereafter. However, the 708s were maintained on the former level until 39-week-old and on the latter level thereafter. The change found at this age; 40-week-old, in level of linoleic acid at 40-week-old might be attributed to two factors; the first is the natural change which is age-related; the second is the change in dietary linoleic acid level, which could be the main contributor. This might be supported by the difference found in levels of γ-linolenic and dihomo γ-linolenic acids between 308 and 708 at that age. Linoleic acid and γ-linolenic acid contents dropped in 708 from 35-week-old when they were maintained on the 12.0-13.0g linoleic acid/kg. This suggests that 708 breeders were not receiving adequate amounts of linoleic acid. This is consistent with the amount of linoleic acid in 308 throughout the first 15 weeks of production, in spite of the diet change at 35-week-old, which was not found in 708. This is also supported by the ability of 308s to keep high levels of γ-linolenic and dihomo γ-linolenic acids after the transition to lower level of linoleic acid at 35-week-old, whereas the 708s were not.

Both DPA and DHA were significantly less in 708 (P<0.001). This difference, found at most of time points studied, could be attributed to the

significant difference in availability of α-linolenic, the substrate for both, which was less in 708 (P=0.005) this makes the contents of n-3 fatty acids in total (except EPA which did not differ significantly) in 708 less than that in their 308 counterparts. Polyunsaturated fatty acids are known to have multiple biological functions; they also play pivotal roles in reproduction. In relation to the current study of importance is their role in oocyte maturation and ovulation. An *in-vitro* study carried out by Mercure and Van Der Kraak (1995) showed that EPA and DHA inhibited steroidogenesis in fully grown but immature ovarian follicles from goldfish and rainbow trout, which is considered an indicator of follicular maturation. Tahara and Yano (2004) investigated fatty acid profiles of the Kuruma prawns ovary at five stages of ovarian development, their results showed that the ratio of n-3/n-6 fatty acids was relatively constant throughout ovarian development stages; as it ranged between (0.75 and 0.85 wt.%), but the maturation stage where it increased significantly to 2.10 wt.%. Ishak *et al.* (2008), who studied fatty acid profiles in ovarian follicles of zebrafish at different stages of development, reported that DHA level was significantly higher in the premature and mature follicles, and linoleic acid was significantly higher in the ovulated follicles.

In conclusion, many observations can be derived from studying liver fatty acid profiles in regard to essential fatty acids, and long chain PUFA. In regard to the essential fatty acid, linoleic acid, there was a significant age by strain interaction, notably, at 40-week-old as it was less in 708. α-linolenic acid was significantly less in 708 with a significant age by strain interaction with the less contents at 25-, 40-, and 45-week-old. Long chain PUFA; there have been a significant age by strain effect on arachidonic acid content as

708 livers had less content at 35- and 45-week-old. γ-linolenic was less in 708 with high difference at 35-week-old, 40-, and 45-week-old. Dihomo γ-linolenic was also less at 40- and 45-week-old. DPA and DHA were significantly less in 708 with a significant age by strain interaction throughout early- to mid-lay. Consequently, the total content of n-3 fatty acids was less in 708 livers, in addition to the differences in n-6 fatty acids this can impair n-3/n-6 fatty acids ratio which might impair follicular development and maturation. Thus, n-3 fatty acids were less with the least contents at 25-, 40-, and 45-week-old; this might suggest impairment to egg production at these ages. By bearing in mind the importance of the n-3 fatty acids for normal follicular development and maturation, this group differed at 25-week-old which might explain the variation in egg production found between flocks from which the experiment breeders were sampled (Figure 3.1). They were also different at 40- and 45-week-old in conjunction with the n-6 fatty acids; these might be linked to drop in production if repeated in other flocks.

Taking these findings into consideration, two conclusions can be drawn from the current observations. The first, whether or not 708 breeders are receiving the adequate level of linoleic acid should be reviewed, the second is that 708 breeders do not seem to be receiving adequate level of α-linolenic acid. Accordingly, modification in the diet of the 708 breeders might be required to meet their needs as well as the best timing for diets transition.

Chapter 5: Body Weight and Fat Content

5.1 Introduction

Although being domesticated for thousands of years; it is only during the second half of the twentieth century that more attention has been given to selective breeding of chickens. With the increasing demand for animal products, chicken meat production has become the most rapidly growing sector among meat species.

Food overconsumption in broilers is the direct result of half a century of selective breeding for growth rate, which has altered the appetite control centres (detailed in Chapter 1; 1.4). This has led to a dramatic increase in appetite; as these genetically developed meat stocks were found to have a greater number of meals as well as consuming more feed in comparison to layer stocks (Masic *et al.*, 1974; Marks, 1980; Barbato *et al.*, 1982; Hocking *et al.*, 1997).

Although growth rate has been enhanced and the modern broiler grows 4.6 times that of those birds bred in 1957 (Havenstein*et al.*, 2003 a), this genetically enhanced growth rate was coupled with impaired reproductive fitness and many undesirable biological dysfunctions detailed in Chapter 1; 1.4.1) such as obesity (McCarthy & Siegel, 1983; Siegel, 1984).

Many researchers have shown that the increased body weight achieved is mainly attributed to increased body fat content, as fat level was significantly decreased when birds were feed restricted. Katanbaf *et al.* (1989c) reported that carcasses from *ad libitum*-fed broiler breeder females

contained around 81 to 110 g/kg more lipids when compared to those from feed restricted birds (Katanbaf *et al.*, 1989c). Another finding revealed feed restricting broiler breeder females during the rearing period has reduced body fat by 8 times compared to the full-fed females at 18-week-old (Yu *et al.*, 1992a). Furthermore, Robinson *et al.* (1991a) found that feed-restricted broiler breeder females contained 369 g less fat when compared to their *ad libitum*-fed counterparts, Renema *et al.* (1999 a) found that their experimental birds contained 77.2 g/kg more lipid when fed *ad libitum* from photostimulation in comparison to those which were restricted.

As two distinct strains, many differences have been addressed between egg and meat type chickens regarding body weight and reproductive performance (Williams & Sharp, 1978 a; Yu *et al.*, 1992 a, b). In this context, the aim of the current study was to investigate the differences between the two broiler breeder strains, 308 and 708, in regard to body weight and fat content. The 308 represents a standard broiler breeder strain and 708 is the result of ongoing genetic selection for more growth traits, which might suggest differences in their metabolic hormone levels leading to variation in their reproductive performance.

Dead body weight of birds was recorded after euthanasia, and thereafter carcasses were processed for fat determination as described in Chapter 2 (2.3.3), total carcass fat content was determined using methods described in Chapter 2 (2.4.3). Results were analyzed as described in Chapter 2 (2.6).

5.2 Results

5.2.1 Flock data

Flock body weight profiles and standard breed body weights for both strains are presented in Figures 5.1 and 5.2. Compared with standard breed weights shown in Parent Stock Management Manuals for Ross 308 and 708, actual flock body weights provided by the company do not seem to follow target body weights for both strains. Females from 308 were 0.06 lighter than standard breed weights, except at 25-week-old when they were 0.05 heavier, whereas females from 708 were 0.11 heavier than they should be. This increase in body weight was associated with greater feed consumption in 708, and in 308 at 25-week-old as they were heavier than standard breed weight. However, from 30-week-old onwards, feed consumption by 308s followed standard feed intake targets. Flock daily feed intakes, as well as standard daily feed intakes, for both strains are presented in Figures 5.3 and 5.4.

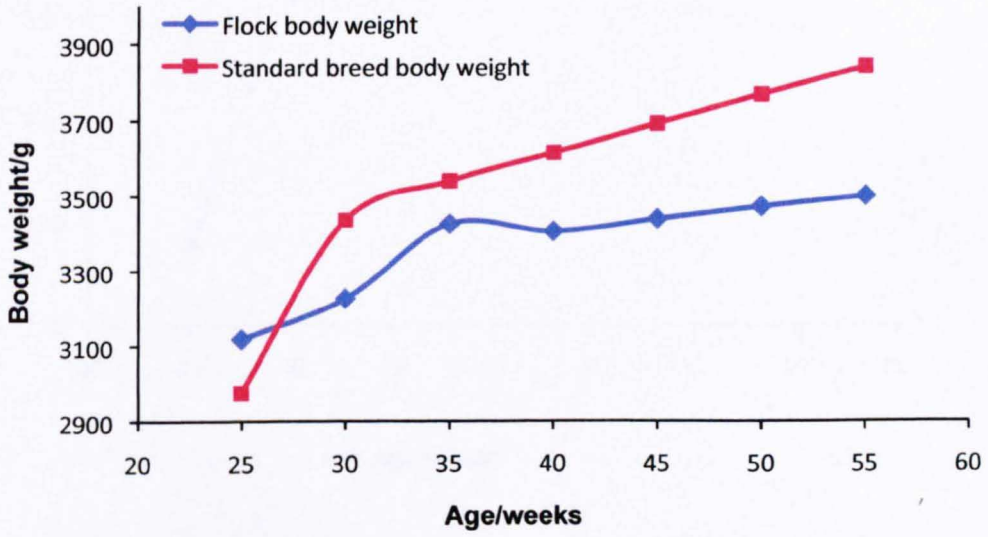


Figure 5.1 Flock body weight profiles and standard breed weight for Ross 308 broiler breeder females (PD Hook & Ross 308 Parent Stock Management Manual).

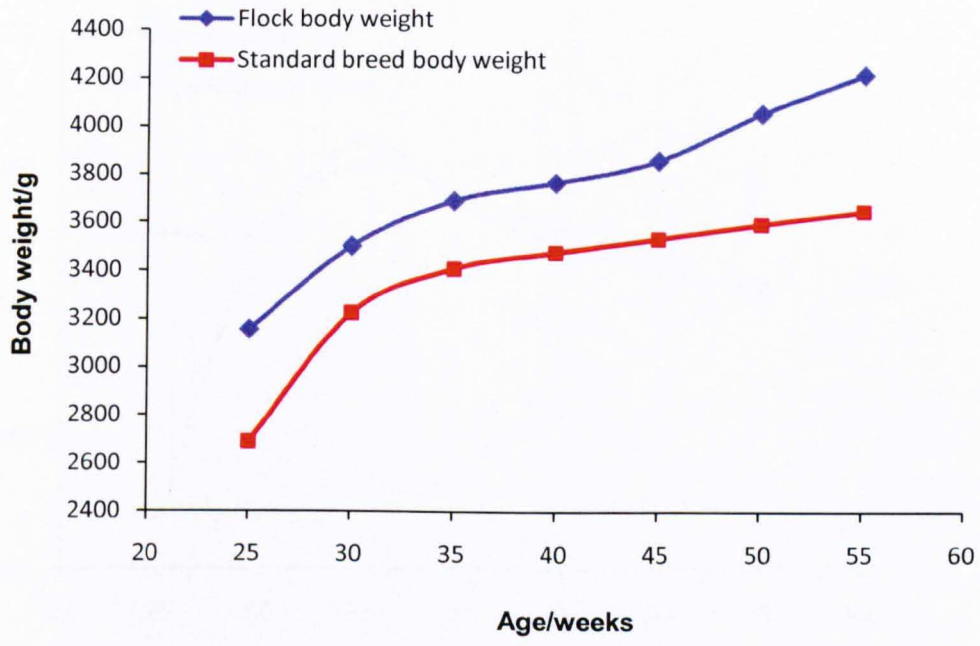


Figure 5.2 Flock body weight profiles and standard breed weight for Ross 708 broiler breeder females (PD Hook & Ross 708 Parent Stock Management Manual).

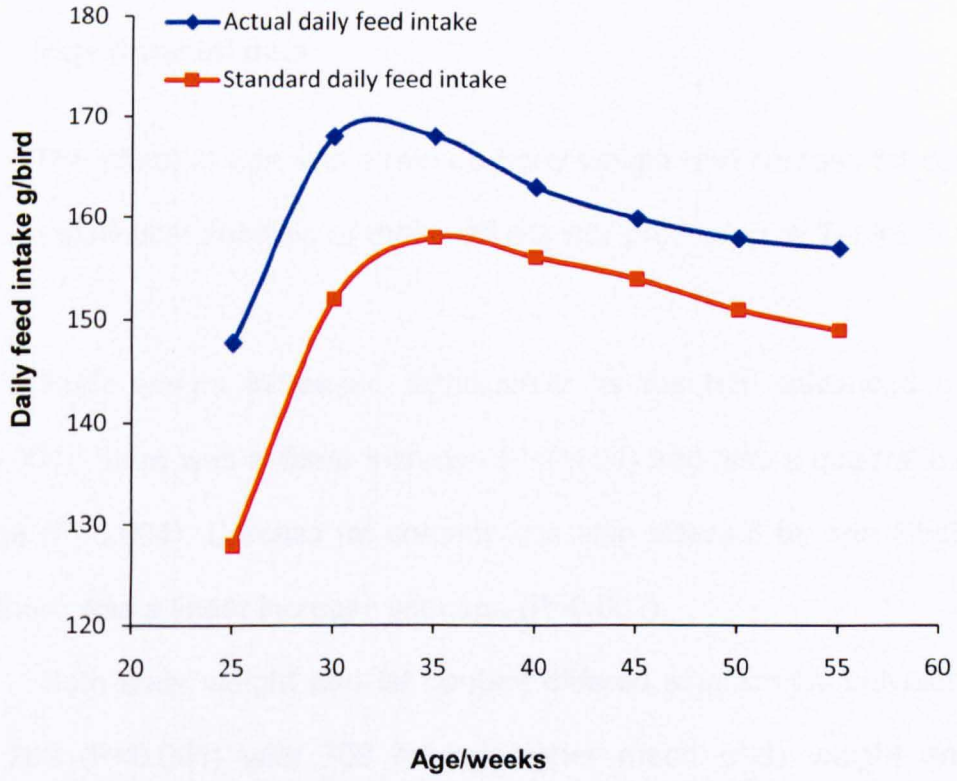


Figure 5.3 Actual and standard feed intake g/bird/day of Ross 708 broiler breeder females (PD Hook & Ross 708 Parent Stock Management Manual).

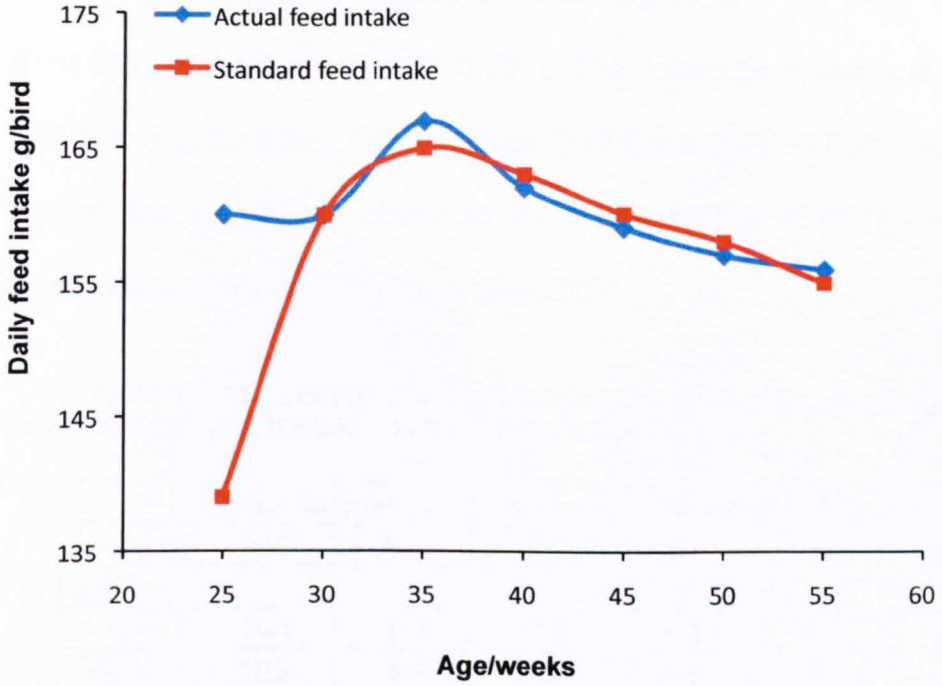


Figure 5.4 Actual and standard feed intake g/bird/day of Ross 308 broiler breeder females (PD Hook & Ross 308 Parent Stock Management Manual).

5.2.2 Experimental data

The effect of age and strain on body weight and carcass fat content, and the statistical analysis of these effects are presented in Tables 5.1 and 5.2.

Body weight increased significantly as the hen advanced in age ($P<0.001$); there was a linear increase ($P<0.001$) and also a quadratic effect of age ($P=0.004$). Carcass fat content was also affected by age ($P=0.015$) and there was a linear increase with age ($P=0.002$).

Both body weight and fat content differed significantly between 308 and 708 ($P<0.001$) with 708 having higher mean body weight and fat content.

Age by strain interaction was significant for both body weight (P=0.002) and fat content (P<0.001). This interaction was linear (P<0.001) with a strong tendency for deviations from linearity (P=0.053) regarding body weight; the same was observed for fat content (P<0.001), however, with significant deviations from linear (P=0.006).

Table 5.1 Effect of age (weeks) and strain on dead body weight (g) and carcass fat content (g/kg) of the 308 & 708 broiler breeder females

	Dead body weight		Mean	Carcass fat content		Mean
Strain	308	708		308	708	
Age						
25	3260	3064	3162	270	270	270
30	3280	3612	3446	286	324	305
35	3632	3838	3735	344	237	290
40	3762	3937	3850	296	409	353
45	3531	3886	3709	294	405	349
50	3430	4488	3959	249	379	314
55	3586	4104	3845	300	409	354
Mean	3497	3847	3672	291	347	319

Table 5.2 Analysis of Variance; showing the effect of age and strain on dead body weight and carcass fat content of the 308 & 708 broiler breeder females

	Dead body weight		Carcass fat	
Factor	SED	P	SED	P
Age	135.7	<0.001	27.6	<0.015
		<0.001 (Lin)		0.002 (Lin)
		0.004 (Quad)		0.251 (Quad)
		0.348 (Dev)		0.21 (Dev)
Strain	72.5	<0.001	14.8	<0.001
Age * Strain	191.9	0.002	39.1	<0.001
		<0.001 (Lin)		<0.001 (Lin)
		0.662 (Quad)		0.811(Quad)
		0.053 (Dev)		0.06 (Dev)

5.3 Discussion

Broiler breeder strains developed for rapid growth have lost the ability to self-regulate feed intake; this has resulted in obesity and consequently undesirable impacts on reproductive performance (detailed in Chapter 1, 1.4). Because these birds are required to carry the genetic material for rapid growth as well as being good layers, human intervention is required to circumvent this negative relationship. Quantitative feed restriction has been found to improve the reproductive performance of the broiler breeders. However, there are different opinions regarding the level of restriction (Wilson & Harms, 1986; Fattori *et al.*, 1991) and the best timing of restriction (detailed in Chapter 1, 1.4.2). Accordingly feeding programmes have been designed to achieve "target" weights.

In the current study, results showed that there was a significant increase in both body weight and fat content with age. These relationships are most probably attributed to the 708 strain as there was a significant effect of strain ($P<0.001$). When comparing the relationships between age and these two parameters in 308 and 708 separately differences can be seen. The relationships between age and both body weight and fat content in 308 were poor (R^2 values were 0.24 for body weight and 0.008 for fat content); whereas they were stronger in 708 (R^2 values were 0.75 for body weight and 0.57 for fat content). This could be related to the different rates at which these two parameters changed over time in both strains. Regarding body weight, 708 birds increased by 1424 g, whereas 308 birds increased by only 502 g. Part of this relationship was related to the fact that body weight at any point of life is made up weight of the different body parts which show

multiphasic growth. Additionally, 708s typical body weight profile, provided by the company, (Figure 5.2) clearly shows that the 708s used in this study were above the standard breed weight throughout their life. This was accompanied by higher daily feed intake compared to the standard daily feed intake. It is likely that this difference was sufficiently large to affect some of the parameters under investigation.

The current study was undertaken on the dead body weight. It is, therefore, of particular importance to mention that birds from both strains were offered *ad-lib* feed for 15-18 hours prior to slaughter; as they were housed at Sutton Bonington overnight till they were euthanized. This could, in part, account for the fact that birds were heavier than typical flock body weights. This might have contributed, to some extent, to this increase which ranged between 55 and 357 g in 308 birds. For 708 experimental birds, there was an additional contributor to their high weights; which ranged between 22 and 422 g at 50-week-old; by comparing dates of birds' arrival with dates and ages in the flock data provided by the company, it was found that 708 were a week older than anticipated, except at 25-week-old. This highlights the issues associated with this type of study, where birds are sourced directly from commercial producers rather than reared under carefully controlled experimental conditions. However, according to the standard weekly weight targets, this would constitute a small proportion of the increase found in body weights which were 55 g from 30 to 31-week-old and 10-15 g thereafter, whereas a considerable contribution can be given to the *ad-lib* feeding.

Fat content also changed at different rates in the two strains; 308 increased by 94 g/kg, 708 increased by 172 g/kg. The significantly higher

content of fat of 708, which had the same fat content as the 308 birds at 25-week-old in spite of having lighter weight, shows clearly the propensity of 708s to lay down more fat. Earlier studies have reported differences in fat content between genetically different lines. In a comparison between four commercial broiler strain crosses, differences in abdominal and carcass fat contents were reported; as the former was found to range between 23.5 and 27.6 g/kg and the latter ranged between 449 and 479 g/kg dry matter (Griffiths *et al.*, 1977). Hood and Pym (1980) compared abdominal fat weight and body fat content in lines selected for different traits, differences in abdominal fat weight ranged between 114 and 162 g/kg, and differences in body fat content ranged between 16.6 and 30.9 g/kg; body weight ranged between 1535 and 1955 g.

Calabotta *et al.* (1983) suggested that differences in fat content between low- and high-weight selected lines could be attributed to differences in lipolysis rather than to lipogenesis activity. As their findings revealed that low-weight selected line birds tended to mobilize more free fatty acids from adipose tissue which was determined by measuring plasma free fatty acids concentration, which was higher compared to that of high-weight selected line. Many studies suggested that hormones might underlie these differenced in lipogenesis and lipolysis activity. Calabotta *et al.* (1983) stated that in a preliminary study, low-weight selected birds had higher level of growth hormone (GH) compared to high-weight selected ones which has been shown to stimulate lipolysis (Harvey *et al.*, 1977 cited in Calabotta *et al.*, 1983). An earlier study conducted by Jones *et al.* (1980) to investigate plasma GH in White leghorn and meat-type chick found that GH level was

higher in the former. Broilers selected for an increased growth had lower level of GH when compared to that of relaxed selection and White Leghorn birds (Goddard *et al.*, 1988).

Ongoing genetic selection for growth traits resulted in a genetic tendency for chickens to fatten (Zubair & Leeson, 1996; Siegel, 1999; Renema & Robinson, 2004). Lonergan *et al.* (2003) studied 5 different stocks and their results showed that broilers were heavier and with the highest weight of breast muscle which also contained more lipid. Results of the current study showed strain differences in fat content. The 708, selected for greater yield of breast meat, was found to lay down more fat compared to 308.

Adipogenesis is a two sequential stages process; during the first adipocyte precursor cells proliferate and differentiate (hyperplasia). This stage takes place early in life and at this stage the complete number of adipocytes is established and is believed to be genetically determined. In the second stage the existing mature adipocytes are filled with lipid (hypertrophy) and this takes place in the adult phase. Considerable investigations have confirmed differences in fatness between genetically different stocks (Littlefield, 1972; Farr *et al.*, 1977; March & Hansen, 1977; Griffiths *et al.*, 1978; Calabotta *et al.*, 1982). March & Hansen (1977) reported that number of adipocytes in White Leghorn chicks was half of that found in broiler-type chicks. Studies were then undertaken to control fat deposition by feed restricting birds early in their life when adipocytes are established. Some results revealed that after 6 days of feed restriction birds were able to compensate growth and achieve complete recovery of body weight, whereas

a 12-days restriction did not allow any body weight recovery (Plavnik *et al.*, 1986). Mollison *et al.* (1984) showed that feed restricting broilers to 90% of the control group from 2 to 7 week of age significantly reduced their weight at 8 week of age. However, being undertaken on broiler chickens and with only one week between the end of restriction and slaughter, it was suggested that the birds might not have had enough time to complete growth compensation. A pioneering study of March and Hansen (1977) where they investigated the development of retroperitoneal depots found that 3 days feed withheld either immediately after hatch or at 10 days old, resulted in retroperitoneal adipocytes being able to proliferate after re-feeding when feed was withheld immediately after hatch. However, this did not occur immediately when feed was withheld at 10-days-old. They also found that cell multiplication continued for a few weeks after hatching and, at the end of their experiment both the control and restricted birds had the same amount of adipocyte DNA. However, the restricted birds had lower fat content.

In the current study, although 708 had lighter weight at 25-week-old in comparison to 308, 708 carcass contained more fat than 308. This might suggest that 708 had developed its complete fat depots (number) which are genetically determined and even started depositing fat earlier than 308. Hood & Pym (1980) stated that fast growing birds might attain their final number of adipocytes earlier than slow growing birds. Thus, it can be concluded that the feed restriction programme used succeeded in controlling body weight and fat content in 308; which is clearly seen as a low relationship between age and both body weight and fat content. However, the good relationship between age and both body weight and fat content found in 708 indicates

that these parameters were not properly controlled. Another point that might be of importance is that 708s were maintained on grower 2 diet for 13 weeks, whereas 308s were maintained on grower 1 for 5 weeks thereafter on grower 2 for 8 weeks, and that grower 2 contained less protein than grower 1 with the same level of ME. In fact, 708 are bred for greater growth and they have the potential for being heavier than 308, thus their maintenance and growth requirements might be different. Accordingly, the difference found between 308 and 708 regarding body weight and fat content at 25-week-old could have provided evidence for the propensity of 708 to lay down more fat; however, diet might have contributed to this level of fat content.

Differences in fat content between strains might reflect differences in metabolic hormones level such as growth hormone, which decreases with continuing genetic selection and is known to stimulate lipolysis in addition to its possible interaction with other metabolic hormones such as thyroid hormones (Calabotta *et al.*, 1983; Goddard *et al.*, 1988; Siegel & Wolford, 2003). Leptin is another metabolic hormone that was found to have both positive and negative effects on reproduction. Paczoska-Eliasiewicz *et al.*, (2003) found that leptin treatment of Hy-Line Brown hens attenuated ovarian deterioration and a decrease in progesterone and oestradiol levels resulted from feed withdrawal. They also found that continuous leptin treatment during re-feeding significantly impeded follicle entry into the hierarchy. These results were in agreement with similar studies undertaken on mammals as it was shown that leptin treatment attenuated reductions in gonadotropins, changes in gonadal axis and also reduced the delay of ovulation resulting from starvation. However, leptin excess was found to affect granulosa cells

steroidogenesis, and follicle maturation *in vitro* (Spicer & Francisco 1997; Nagatani *et al.*, 1998; Ahima &Flier, 2000; Kikuchi *et al.*, 2001). Therefore, it can be said that there is a threshold for leptin that separates its positive and negative effects, and that successful feeding programmes attain that crucial level. In the current study, feed intake of both strains was similar, except at 25- and 30-week-old where 708s feed consumption increased from lower to higher daily consumption than the 308s which might have resulted in differences in metabolic hormones.

In conclusion, many factors seem to contribute to the higher fat content found in 708 carcasses. Firstly, 308s were under standard breed weight, but 708s were over standard breed weight; secondly, there were variations in the diet (detailed above). However, whether ongoing genetic selection contributed to this higher fat content cannot be certain under the current circumstances, but it has been suggested that 708s have established their fat reservoirs earlier than 308s, which can be attenuated by timing of feed restriction.

Chapter 6: Tibia Bone and Calcium Content

6.1 Introduction

Calcium is a major element for egg production in the laying hen. Since the early decades of the twentieth century the importance of this element has been well documented, as low levels of dietary Ca resulted in a significant reduction in egg production. Furthermore, Ca exclusion from the diet and limiting it to the natural contents of the diet led to the cessation of egg-laying earlier than birds whose diets were supplemented with Ca (Buckner & Martin, 1920). Hughes *et al.* (1927) found differences in blood calcium concentration between immature, mature; non-laying and laying-hens (13, 20, and 27 mg/100cc, respectively). Another study reported 20% difference in Ca level between arterial and venous blood during shell calcification, with the artery having the higher content (Winget *et al.*, 1958). Calcium constitutes around one third of egg-shell weight, which highlights the importance of Ca in the egg production process. The required calcium is provided by the cooperation of the intestine, kidney, and the medullary bones, as the hen use three strategies for this purpose; first, increasing intestinal absorption of Ca; second, reducing Ca loss via excreta by the kidney; and third, mobilizing Ca from the medullary bones.

Calcium level is controlled by three hormones, parathyroid, calcitonin, and oestrogen, in addition to 1, 25 dihydroxycholecalciferol the active metabolite of vitamin D. Cholecalciferol (D_3) is the active form of vitamin D that is used by birds, and is metabolized in both liver and kidney. In

the hepatic microsomes, D_3 is metabolized to 25-hydroxycholecalciferol 25-OHD_3 by the mediation of cholecalciferol -25 hydroxylase enzyme. In the renal mitochondria, (25-OHD_3) is then mobilized to 1,25 dihydroxycholecalciferol (1,25 $(OH)_2D_3$) by the mediation of 25-hydroxycholecalciferol 1α-hydroxylase enzyme. The latter synthesized form of vitamin D is of a key role in Ca metabolism (Lund & DeLuca, 1966; Haussler *et al.*, 1968), as it regulates the intestinal absorption of Ca via instigation of RNA transcription and synthesis of calbindin D_{28k} protein which possesses a high affinity to bind Ca (Christakos *et al.*, 1989; Norman *et al.*, 1992), as well as increasing Ca mobilization from the medullary bone (detailed in chapter 1, 1.9.2). Regulation of Ca level by this system is also detailed in Chapter 1 (1.9.2).

Many studies have shown the role of long bones as reservoirs that supply Ca for shell formation during the night when no feed is provided (Chapter 1, 1.9). In the light of this, it was proposed to investigate the dynamic of this element in the tibia bone to identify any differences between 308 and 708 in utilizing this reservoir that might underlie the differences in their reproductive performance.

Bones were processed and prepared for Ca analysis as described in Chapter 2 (2.4.4). Results were analyzed as described in Chapter 2 (2.6).

6.2 Results

The effect of age on tibial contents of Ca in both strains and the statistical analysis of these effects are presented in Tables 6.1 and 6.2.

Tibia content of Ca was significantly (P<0.001) affected by age, as there was a linear decrease P< (0.001) followed by an increase (quadratic; P< 0.001), there was also an effect that is beyond quadratic (Dev; P<0.001).

There was a trend (P=0.087) for the 708s female towards having lower tibial content of Ca. There was a significant effect for age by strain interaction (P<0.001), this effect of interaction was linear (P<0.001); as the 708s tibiae contained less Ca over early- and until mid-lay.

Table 6.1 Influence of age (weeks) and strain on the tibial content of Ca (g/kg) in 308 & 708 broiler breeder females

			Mean
Strain	308	708	
Age			
25	380	373	377
30	364	358	361
35	362	357	359
40	354	358	356
45	355	355	355
50	362	362	362
55	361	363	362
Mean	363	361	362

Table 6.2 Analysis of variance; showing the effect of age and strain on the tibial content of Ca in 308 & 708 broiler breeder females

Factor	SED	P
Age	1.8	<0.001
		<0.001 (Lin)
		<0.001 (Quad)
		<0.001 (Dev)
Strain	0.9	0.087
Age * Strain	2.5	<0.001
		<0.001 (Lin)
		0.328 (Quad)
		0.259 (Dev)

6.3 Discussion

Many changes have been associated with advanced age that affects egg production. Abe *et al.* (1982) investigated cholecalciferol metabolism in egg laying hens at different ages, and confirmed that (1,25 $(OH)_2$ D_3) synthesis decreased as the hen ages, and that even injection of (1,25 $(OH)_2D_3$) did not result in significant incorporation into the target tissues, duodenum and bones (femora and tibiae), suggesting a decrease in the binding capacity of the receptor for (1,25 $(OH)_2D_3$). A third observation is that even after injection of this metabolite into older hens they still had lower levels of it in their plasma and that led the researchers to conclude that the rate at which this metabolite is degraded is accelerated with advanced ages. Elaroussi *et al.* (1994) reported a significant decrease in activity of renal 1α-hydroxylase enzyme in old hens compared to young ones. Also, they reported a different response in old and young hens to Ca restriction in terms of increased levels of 1,25 $(OH)_2D_3$, as old hens had less capability to adapt to the restriction; thus their observations suggest a reduced capacity to mobilize Ca from medullary bone as the hen ages. Bar & Hurwitz (1987) found that when comparing normal and low Ca diets for commercial egg laying hens at 9 & 21 months of age, tibia ash was lower in the low Ca diet at both ages, which they attributed to increased Ca resorption from the bone. Tibia ash of the normal and low Ca diets was 2841 and 2433 mg/bone in young hens, whereas it was 3073 and 2631 mg/bone in old hens. Albatshan *et al.* (1994) also found that *in vitro* calcium uptake by the duodenum of egg laying breeders at different ages decreased between 37 and 58 weeks of

age. Thus, these findings indicate reduced Ca absorption and utilization from the long bone by aged hens.

In the current study there was a significant linear effect of age as the tibial content of Ca decreased over early to mid-lay, then there was a significant increase (quadratic effect) at late-lay. This latter increase could indicate bone Ca replenishment rather than mobilization, which, in turn, reflects the reduced need for the female to use Ca from the tibia bone.

Females from both strains utilized the tibial Ca reservoir at the same rate over early- to mid-lay and both commenced to replenish tibia bone calcium at around mid-lay. There was an initial difference in Ca content between both strains at 25-week-old which continued over a few weeks. The similar content of tibial Ca in both strains from mid-lay and onwards, in spite of the fact that 708 had less Ca contents at younger ages, leads to speculation that reduced availability of Ca for egg formation might affect the rate of egg laying.

In a study of dietary manipulation during laying period, Roland (1979) found that reducing dietary protein from 160-115 g/kg resulted in a reduction in serum Ca by 2.7 mg/dl. And that increasing dietary Ca level with low dietary protein increased the reduction of Ca serum to 4.7 mg/dl. In the current study, both strains were fed the same level of Ca during rearing and pre-breeder periods, until 21-week-old, except that 708 females were maintained on grower 2 diet for 13 weeks (6 to18 weeks of age). This diet contained less protein and amino acids, total lysine, and total methionine plus cystine, whereas 308 females were maintained on this diet for 8 weeks only. It might be possible that being maintained on lower protein and amino

acids might have impaired Ca level and resulted in less tibial Ca seen in 708 over early-lay. Another observation is that 708s were also fed a diet of less Ca content, breeder 1, for 4 weeks longer than their 308 counterparts (see Tables 2.2 & 2.4 in Chapter 2).

Compared with strain 308, 708 entered the laying cycle with less Ca reserves, which continued over the first 15 weeks of egg production as there was a significant age by strain effect. Whether or not this affected 708s egg production, and whether or not these dietary levels of protein, amino acids and Ca meet 708 requirements, more investigation is required as the entire rearing period is crucial for subsequent laying performance. In fact, these findings suggest a possible variation in egg production at early-lay. However, utilizing Ca at the same rate by the breeders of both strains thereafter might suggest a similarity in egg production. This might be consistent with the actual egg production profiles provided by PD Hook. However, no evidence can be provided to support this because egg laying sequences were not measured, as birds were reared on the commercial farms rather than under carefully controlled experimental conditions.

Chapter 7: General Discussion

Over the last half a century, genetic selection has continued in the chicken meat sector for greater growth. This selection, centred on growth traits rather than reproduction, has led to an imbalance between growth and reproduction. This negative relationship was firstly identified between egg-laying and meat-type breeders and it seems not to be only limited to these two distinctive lines, but it also exists between meat-type strains. In the current study females from two broiler breeder strains, Ross 308 and 708, were studied as these two strains were found to differ in their reproductive performance. Thus, this study aimed to identify fundamental differences that might underlie the reduced reproductive performance of 708 females. Four key points were investigated; follicle numbers, liver fatty acid profiles, body weight and fat content, and finally tibia content of calcium.

Findings of this study revealed differences in the key points studied between females from the both strains. However, investigating these points together should give a clearer insight into the differences underling differences in their reproductive success.

7.1 Effect of age

Age had a significant effect on the parameters studied. Follicle number was found to be significantly affected by hen age; both large yellow follicles (LYFs) and small yellow follicles (SYFs) were found to decrease significantly with advanced age. In contrast, large white follicles (LWFs) were found to increase significantly as the hen advanced in age. Largest follicle

diameter was found to increase significantly with age. Liver fatty acid contents were found to decrease with age, the same was also found with regard to tibial content of calcium. However, both body weight and carcass fat content were found to increase significantly with age.

Many changes occur as the hen advances in age; this could be summarized in the following. As the hen advances in age, capability of synthesizing fatty acids by the liver is reduced as a consequence of changes in the fine structure in the endoplasmic reticulum, and also responsiveness of hepatocytes to lipogenesis agonists was found to decrease as the chickens advanced in age (Schmucker & Jones, 1975; Schmucker, 1976; Harris *et al.*, 1988). Because the liver is the main site of fatty acid synthesis, that means a reduction in the fatty acids available for distribution to all body tissues. The increasing fat content in broiler breeder strains as the hen advanced in age (Robinson *et al.*, 1991a; Zubair & Leeson, 1996; Siegel, 1999; Lonergan *et al.* 2003; Renema & Robinson, 2004) indicates that more fatty acids were allocated to fat depots making less available to the follicles, and a strategy followed by the hen is to accumulate fatty acids available in fewer follicles (Williams & Sharp; 1978). In the current study there was a significant decrease in the amounts of most fatty acids with advanced age. Thus, increased follicle diameter with advanced age could be an adaptive strategy, by the hen, to reduced fatty acid synthesis with advanced age in order to render adequate levels of fatty acids that are important for embryo growth and development. Nielsen (1998) stated that the high percentage of long chain polyunsaturated fatty acids in eggs from young hens might be a compensation for small yolks, as yolk packaging represents the maternal role

providing adequate nutrients for a fully developed chicken embryo. Thus, the opposite view, that larger yolks from older hens is a compensation for the inability to provide adequate levels of these fatty acids with advanced age, could be possible. However, it would be an interesting point of investigation to study levels of different fatty acid classes in the largest follicles at different ages. This was not undertaken in the current study, and fatty acid profiles were investigated in the liver not in the follicles since the liver is the site that reflects dietary fatty acid contents.

Many factors seem to contribute to the reduction in the LYFs number. A first contributor is the LYF itself, which seems to become less sensitive to gonadotrophins (Berry & Brake, 1985; Decuypere & Verheyen, 1986) and the delay in its differentiation, and consequently its maturation as well as the longer time taken for its growth has been reported (Moudgal & Razdan, 1985; Johnson *et al.*, 1986; Palmer & Bahr, 1992). Subsequently, this might result in a shortening of the egg laying sequence and increasing number of pauses days; thus, reducing egg production. The reduced absorption of Ca with advanced age (Abe *et al.*, 1982) as well as reduced ability to mobilize Ca from medullary bone (Elaroussi *et al.*, 1994; Bar & Hurwitz, 1987), both contribute to the reduction in Ca available for vitellogenin; thus a possible delay in follicular growth, and for calcification. This also might affect the proliferation of the granulosa cells which have been found to have calcium-sensing receptors; of importance for cell proliferation; thus changes in Ca availability with advanced age may contribute to granulosa cells apoptosis (Diez-Fraile *et al.*, 2010) and, therefore, the possibility of LYFs atresia which could be found at advanced ages (Berry & Brake, 1985; Verheyen, 1987).

Many other factors participate in the termination of the first egg-laying cycle; but they were beyond the scope of the current study.

7.2 Effect of strain

Results of the current study have identified differences between 308 and 708 in some of the studied parameters. Linking these parameters together would enable better understanding of the differences in their reproductive performance.

7.2.1 Large white follicles number

LWFs differed significantly between 308 and 708, in addition to a significant age by strain interaction. This difference was found at 35-, 40-, 50-, and 55-week-old. There is little information in the literature regarding this type of follicle; however, this type of follicle has been found to increase with age and their number has been found to differ between strains (detailed in Chapter 3; 3.3).

Some studies that investigated the effect of feed allowances on ovarian function, found that the number of the LYFs was related to body weight (Hocking & Whitehead, 1990; Hocking, 1996). This might suggest that such a relationship between body weight and number of large white follicles exists, leading to impairment of reproductive performance as a direct result of the increased body weight. As the increased number of this type of follicle take place at advanced ages concomitantly with reductions in LH and FSH levels, it could be possible that steroids produced by these follicles feed negatively back to the hypothalamus and pituitary. It was not possible to investigate this in the current study because body weights of both strains did

not follow standard breed body weights, and deterioration of blood samples prevented FSH assay.

7.2.2 Liver fatty acid contents, large yellow follicle numbers and fat content

Two points will be discussed; the total content of fatty acids, and the possible effect of n-3 fatty acids on follicle development and egg production.

Total liver fatty acid content and LYFs did not differ between strains, whereas 708 had a significantly higher carcass fat content. By taking into consideration that the liver is the main site that supplies tissues with fatty acids; it could be assumed, according to the first finding, that livers of both of 308s and 708s; which did not differ significantly in their total fatty acid contents, supplied almost the same number of LYFs. Whereas, having a higher carcass fat content, the 708s might be allocating more fatty acids to adipose tissue. This was noticed at 25-week-old as 308 and 708 consumed the same amount of feed over the standard daily feed intake; according to the information provided by PD Hook, yet the 708s showed lower egg production at this age and the same fat content in spite of having the same number of follicles and lower body weight. This might indicate that the 708s utilized the available feed for fat deposition, whereas the 308s utilized the same amount of feed for egg production. At 30-week-old, the 308s consumed the same as the standard daily feed consumption whereas the 708s consumed more than the standard daily consumption and more than the 308s which caused 708s to attenuate egg production, but still less than the 308, and also to lay down more fat (Figures 3.1, 5.3, 5.4). These two

observations might show that, when feed is available, the priority of 708s is to deposit fat ahead of egg production.

The "Resource Allocation Theory" was first suggested by Rendel (1963; cited in Decuypere *et al.*, 2010) who found that the negative genetic relationship between two traits is the result of their competition for resources. In the light of this, fitness can be achieved by partitioning resources intermediately between different traits; however, when resources are allocated to a particular trait, then, bird fitness (health, maintenance, reproduction, etc.) is reduced. Basically, that happens under continuing selection for greater growth, thus the supply of resources will be greater for growth rather than other traits (Goddard & Beilharz, 1977 cited in Rauw *et al.*, 1998), which could explain the reduced performance of meat-type parent stock. This could apply in the current situation; 708s represent ongoing selection for greater growth in comparison to their 308 counterparts, and they seem to allocate more resources to growth at the expense of reproduction. However, the current study has not been able to provide any evidence regarding the effect of continued genetic selection on the propensity to lay down more fat; there were many factors which might have contributed to the higher fat content of 708s *vs.* that of 308s. According to the flock body weight profiles provided by PD Hook 308 birds were 0.05 under standard breed weight, whereas the 708s were 0.11 over standard breed weight. This discrepancy in the farm production systems was unfortunate and could have, at least in part, affected the comparisons made in the current study. Additionally, 708s were found to be one week older than anticipated ages, except at 25-week-old, which might also explain a proportion of their higher

carcass fat content. 708s reached their highest carcass fat level at 40-, 45-, and 55-week-old; however, this investigation did not include egg production profiles of the experimental birds, so it is not known if this affected egg production. Therefore, it would be difficult to predict how the experimental birds, with this varied fat content and almost the same number of LYFs, partitioned their available fatty acids between fat depots and follicular growth. Despite the reported differences in egg production between the two strains, the actual egg production profiles of the two flocks from which the experimental birds were obtained did not show any difference in egg production at these ages. As discussed elsewhere, the use of a larger experimental sample size would have allowed a more robust confirmation of this discrepancy. These two strains did not show any egg production difference from around 30-week-old onwards, although 708s contained more fat. The 308s, consumed feed at the standard daily feed consumption at 30 weeks of age (Figure 5.3), their egg production was also the same as the standard weekly egg production (Figure 3.2), but was higher that 708 at 30-week-old with no increase in body weight or fat content; this suggests that egg production had priority over fat deposition for the 308s. For the subsequent ages both feed consumption and egg production were the same as standards. Although the 708s had the same egg production as 308 from around 30-week-old onwards; however, this was accompanied by greater feed consumption, which might have enabled 708 females to have the same egg production and also to deposit fat. In practice, 708s standard daily feed intake and standard egg production show that the 708 are supposed to have the same egg production with lower feed intake. However, their real

response towards laying down fat or producing eggs cannot be determined unless they are receiving the standard feed intake.

The second point is the (n-3) fatty acids. 708s had significantly lower contents of α-linolenic acid, DPA and DHA compared to the 308s, although EPA did not differ significantly. Several studies have shown the crucial role n-3 fatty acids in reproduction, specifically, in relation to follicular maturation. These studies revealed two possible roles for fatty acids; firstly, as precursors to eicosanoids (prostaglandins), which were found to increase in accordance to the progression of vitellogenesis (Spaziani *et al.*, 1993; Spaziani & Hinsch 1997), although large quantities of fatty acids did not result in high levels of eicosanoids, which might reflect the involvement of these fatty acids in other reproductive physiological processes; secondly, n-3 fatty acids play a role in follicular maturation by inhibition of follicular steroidogenesis, the most important sign for follicular maturation. Mercure & Van Der Kraak (1995) found that polyunsaturated fatty acids inhibited gonadotrophin-stimulated steroid production by fully-grown pre-maturational ovarian follicles from gold fish and rainbow trout in an *in-vitro* study. They found that inhibition of steroidogenesis by fatty acids could be either via affecting the formation of the protein responsible for the substrate (cholesterol) transition, cyclic adenosine monophosphate, or via affecting availability of the substrate itself. This occurs via regulating expression of enzymes involved in cholesterol metabolism, where polyunsaturated fatty acids act in a hormone-like manner (Johnson & Tilly, 1990). Nevertheless, Ishak *et al.* (2008) reported a high level of linoleic acid in the ovulated follicle. In the current study there was a significant age by strain interaction, notably

at 40-week-old, in addition to an inconsistent change in its level throughout early- to mid-lay. However, no firm conclusion can be drawn as egg production profiles of experimental birds were not measured.

Taken together these findings might suggest possible differences in follicular development and maturation between 308s and 708s, which could lead to differences in egg-sequence length and number of pauses days, and consequently differences in egg production.

7.2.3 Calcium content

The precise role of calcium in reproduction, as a component of the yolk as well as for shell formation, is well documented. Pioneering studies (Urist, 1959; Wood-Gush, 1963; Mehring, 1965) suggested the ability of the hen to estimate calcium loss and to react by reducing laying rate rather than reducing shell deposition. Tibia content of calcium has been investigated as an indicator of the dynamic of this element as medullary bone is the Ca supplier when no feed is available.

In the current study, 708s tended to have a lower tibial content of Ca, and there was a significant age by strain interaction with 708s entered their laying cycle with less Ca reservoir. Whether or not this contributed to any difference in egg laying rate cannot be determined as number of eggs laid or egg laying sequences were not measured in experimental birds. The tendency of 708s to have less tibial Ca could lead to the speculation that differences between strains in Ca available for vitellogenin might affect rate of lay, as vitellogenin is an important component of yolk material, and is soluble in plasma when large amounts of Ca are available as it binds large

amounts of Ca, thus inadequate Ca will reduce the yolk material (Schjeide & Urist, 1956; Urist *et al.*, 1958).

7.3 Responses to feeding programme

Birds were fed standard diets according to PD Hook. Liver fatty acid profiles showed that linoleic acid did not change consistently in 708 as it did in 308, there was a strong trend (0.056) for 708s to have less liver linoleic acid, and there was a significant age by strain interaction at 40-week-old; which is in fact at 41-week-old as these breeders were found to be a week-older than anticipated, which might show the effect of diet transition on liver fatty acid content. This might suggest a possibility of inadequate levels of this linoleic acid. Alpha-linolenic acid, the second essential fatty acid and the substrate for long-chain n-3 fatty acids, was significantly lower in 708s, and there was a significant age by strain interaction with the lowest contents at 25-, 40-, and 45-week-old; this might suggest impaired egg production at these ages. However, there was no evidence provided by the current study, except in the actual flock egg production profiles at 25-week-old.

Body weight and carcass fat content were higher in 708s. Although body weight of the 708s at 25-week-old was lower than that of 308s, body weight did not differ between strains thereafter. The 708s were fed on grower 2 for five weeks longer than their 308 counterparts, which were fed on grower 1 during that time (from 6- to 10-week-old). Grower 2 contained more protein and amino acids, but had the same energy content as grower 1; this might have allowed more fat accumulation and lower body weight in 708s.

Feed restriction timing has been a point of intensive investigations, as the best restriction timing enables better reproductive performance. Hood & Pym (1980) stated that fast growing birds might attain their final number of adipocytes earlier than slow growing birds. According to the present findings, having the same level of fat with less body weight could indicate that 708s have established their fat depots earlier than 308s. As the number of adipocytes is genetically determined, the feed restriction programme, including timing, should delay the time at which hyperplastic growth is completed. A pioneering study undertaken by Pfaff & Austic (1976) showed that feeding diets of either high protein content or low energy content is efficient to attain that delay. They found that restricting Single White Comb Leghorn females from hatch to 9.5-week-old on either of the experimental diets resulted in lower levels of adipose tissue up to 22-week-old. They also found that pullets fed on the low energy diet maintained reduced abdominal fat pad weight for 17 weeks after the experimental diet was discontinued, and this reduction was attributed to a reduced cell size with no effect on cell number. However, the effect of genetic selection on carcass fat content could not be determined in the current study; the 708 breeders were fed a diet which might have allowed fat accumulation; they were also over the standard breed weight. The 308s were also over the standard breed weight at 25-week-old but the 708s were heavier than the 308s.

The lower content for708s of tibial Ca found at the onset of laying-cycle could also be attributed to a diet effect. Roland (1980) found that reducing dietary protein from 160 to 115 g/kg resulted in a reduction in serum Ca by 2.7 mg/dl, and that increasing dietary Ca level with low dietary protein

increased the reduction of Ca serum to 4.7 mg/dl; thus the lower level of protein that 708 received for five weeks during the rearing period could be a reason. Another possible reason could be linoleic acid. Kreutter *et al.* (1983) found, in an *in vitro* study, that calcium uptake into brush-border vesicles was reduced when feeding chicks an essential fatty acid deficient diet for two weeks. Their investigation postulated an alteration in the brush-border membrane lipid structure which affected the response to the 1,25 dihydroxycholecalceferol; as they found a decrease in linoleic acid whereas oleic and arachidonic acids were found to increase. The same observation of the effect of essential fatty acid restriction on Ca transport was also found in an earlier study by Hay *et al.* (1980) on rats. In the current study, 708s had a strong trend (P=0.056) towards having lower linoleic acid with inconsistent levels throughout the first 20 weeks of production, and they had significantly higher oleic acid; since the liver is the main site of fatty acids to the different tissues this could indicate a possible alteration in cell membrane lipids which, in turn, might affect rate of calcium transport.

Diez-Fraile *et al.* (2010) confirmed the presence of calcium-sensing receptors (CaR) in the granulosa cells of the largest preovulatory follicle and also in the granulosa cells of the post ovulated follicle. They found that adding (CaR) activating agonists inhibited GC apoptosis as well as increasing their number, whereas adding (CaR) inhibiting agonists caused GC apoptosis; this could be an indicator of an important role of Ca in inhibiting GCs apoptosis and consequently follicular atresia. However, this might contribute to reduced egg production at advanced ages as large yellow follicle atresia is common. However, it seems that Ca has an important role

in follicular maturation as well. Mercure & Van Der Kraak (1995) stated that the inhibitory effect that polyunsaturated fatty acids have on follicular steroidogenesis could be mediated by a calcium-dependent mechanism.

7.4 Conclusions

In summary this thesis can be drawn to the following conclusions.

7.4.1 General conclusions

- Age had a significant effect on the parameters investigated; body weight and carcass fat content increased significantly as the broiler breeder females advanced in age. Large and small yellow follicle numbers decreased with advanced age in contrast to the LWF number which was found to increase. Most of the fatty acids studied decreased with advanced age. However, tibial content of calcium was found to be higher at later ages.
- Ross 708; a strain developed for more breast-meat yield, was found to have significantly higher body weight as well as to lay down more fat. However, differences in both of these parameters could be attributed to the fact that 308s were which standard breed weight whereas 708s were above standard breed weight.
- Differences in lipid metabolism were observed, as livers of females from both strains were found to have significantly different fatty acid contents under the standard feeding diets used by PD Hook.
- It is possible that deficient diets might have caused the observations regarding ovarian morphology referred to in Chapter 3.

7.4.2 Specific conclusions and future work

- The significance difference found between 308 and 708 regarding the large white follicle requires more investigation, in terms of its relationship to the body weight, and the possible effects it might have on reproductive hormones such as LH and FSH.
- Diets applied to 708s do not seem to meet their needs, in regard to essential fatty acids, as there was a strong trend for having less content of linoleic acid and a significant age by strain interaction, whereas alpha-linolenic acid was significantly less.
- Feeding programme used appeared to control neither body weight nor carcass fat content of the 708s. As the 708s body weight changed within 1424 grams whereas it was within only, 502 grams in the 308s. However, having the same fat content with less body weight at the onset of lay could be caused by either the diet or timing of feed restriction if it is a result of genetic selection.
- These observations lead to the importance of reviewing the diets and refining them to best meet the 708 requirements. It is also important to review feeding restriction programme and timing for better control of both of body weight and fat content and then better reproductive performance of 708 females could be expected.
- Inclusion of fish meal for longer time in 708s rearing diet should be investigated, as the n-3 fatty acids were found to significantly reduce abdominal fat pad mass (Newman *et al.*, 2002) which was postulated to be highly correlated with total fat content (Becker *et al.*, 1979)

- Metabolic hormones should be measured as they reflect physiological status, and might explain differences in reproductive performance.
- Investigating length of egg-sequences and total egg production are also of importance, as they reflect the differences found in the parameters investigated.
- This study was undertaken to investigate the effect of age and strain on the reproductive performance of Ross 308 and 708 broiler breeder females. Findings of the current study have shown how the parameters studied changed with age. However, they were not able to address the effect of strain; the ongoing genetic selection on reproductive performance of the normal breast-yield 308 and the breast-yield selected 708. Many other factors might have contributed to the reported reproductive problem with the 708 breeder females. In fact, this study was undertaken under the standard production system according to PD Hook. Breeders were reared on two different farms, and they did not follow target body weights. Comparing dates accompanied with flock data revealed that 708s were a week older than the 308s might have contributed to differences detected in body weights and fat content; even though it was a small proportion. The main factor that might have contributed to the high body weights of the experimental breeders from both strains is the 15-18 hours of *ad-lib* feeding that breeders were maintained on overnight, which made them even heavier than the typical flock body weights. To avoid this, birds should have been feed-restricted exactly the same as they used to be on their farms; as they were offered feed at 9.30 am only. Diets fed to breeders did not seem to meet the 708

requirements which might, in turn, affect their performance. More frequent sampling of larger numbers of birds would allow more precise determination of these effects. Taking all these factors taken into consideration, it would be useful to conduct the study on more than two strains, and it would be better a comparison of how genetic selection affects egg production if an egg-laying strain was included. It is also necessary to conduct the study on more than one generation and flock, as the egg profiles of the current flock did not show any early egg production decline for the 708s in comparison to their 308 counterparts which has been reported by the PD Hook (Breeders Ltd; Daniel Dring; personal communication). Instead, there were differences in egg production throughout the first four weeks of egg production. Finally, it would be more reliable to rear the breeders on a metabolism unit and keep individual egg production profiles for the breeders studied. However, data obtained under these experimental conditions would not necessarily reflect what would be seen in the commercial situation. The current study made strain one of many variables which affect egg production. Thus, having these factors controlled, and the only variable is strain would enable detection of the effect of continued genetic selection on reproductive performance.

Some studies have demonstrated a genetic correlation between average clutch length and total egg production in the turkey; the realized genetic correlation was 0.41±0.28, (Nestor, 1980). He also reported a positive correlation between average clutch length and maximum clutch length (0.81±0.21) on one hand and a negative correlation with number of

clutches (0.49±0.31), on the other hand. Chen *et al.* (2007) investigated the relationship between egg-production until 50-wk-old and both average clutch length and average pause length across 5 genetically different strains. They found that the high egg production group had significantly longer average clutch length and tended to have shorter average pauses. They were able to correlate some laying traits; average clutch length, average pause length and oviposition lag within clutch and the expression levels of related transcripts in the hypothalamus and pituitary gland. These findings were supported by Shiue *et al.* (2006) who identified several transcripts that were expressed in the hypothalamus and pituitary gland and were related to egg production.

Broiler breeder females, when maintained on the same feed allowances, were found to have different "reproductive response" reflecting their ability to balance between egg production and growth as reported by Renema *et al.* (2006). Some breeders were found to produce eggs well at the expense of growth, others were able to produce eggs well and gain body weight relative to flock average, whereas some breeders neither produced, nor grew well. Therefore, selection of breeders that have the ability to balance between growth and egg production for a few generations, could supply breeders that possess genetics for both growth and reproduction.

Although this study was not able to address the effect of ongoing genetic selection for more growth traits, it was able to address some differences between strains in metabolism. Differences in lipid metabolism indicated inadequate levels of essential fatty acids. There

was also a difference in calcium metabolism throughout early-lay. These might partly explain differences found in egg production at the first four weeks of egg production as egg production profiles provided by PD Hook revealed. However, in spite of differences at 40- and 45-weeks which might have explained the reported early egg production decline, this could not be evidenced as there was no individual egg production profiles of the experimental birds as they might represent the reported reproduction problem with the 708 regardless of the typical flock egg production profiles. Fat content which was found to be higher in the experimental birds might cause differences in metabolic hormones; such as leptin and its possible interaction with other metabolic hormones, which act in concert with reproductive hormones and thereby affect reproductive process. Egg production and feed intake profiles of the actual flocks in comparison to the standards showed that the 308s utilized their feed allowances for egg production at the expense of body weight; however, the 708s had the same egg production but with consuming more feed compared to the standard consumption. Therefore, taking into consideration findings of the current study, which revealed some physiological aspects of the 708s, may enable better management of the 708s, and then the genetic selection effect on reproductive performance could be investigated.

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Appendixes

Appendix A (1)

Feeding allowance (g/b/day) of 308 & 708 broiler breeder females from hatch to 140-day-old

Age/day			Age/day			Age/day			Age/day		
	Feed allowance (g/b/day)			Feed allowance (g/b/day)			Feed allowance (g/b/day)			Feed allowance (g/b/day)	
	308	708		308	708		308	708		308	708
1	25	25	36	45	45	71	61	61	106	71	80
2	18	18	37	45	45	72	61	61	107	71	80
3	18	18	38	46	47	73	61	61	108	71	80
4	20	20	39	46	47	74	61	61	109	71	80
5	20	20	40	46	47	75	61	61	110	71	80
6	24	24	41	47	50	76	61	61	111	71	80
7	24	24	42	47	50	77	61	61	112	71	80
8	26	26	43	47	50	78	63	62	113	71	86
9	26	26	44	50	50	79	63	62	114	76	86
10	28	28	45	50	52	80	63	62	115	76	86
11	28	28	46	50	52	81	63	62	116	76	86
12	30	30	47	54	52	82	63	62	117	76	86
13	30	30	48	54	52	83	63	62	118	76	86
14	32	31	49	54	52	84	63	62	119	76	86
15	32	31	50	55	55	85	64	63	120	82	92
16	34	32	51	55	55	86	64	63	121	82	92
17	34	32	52	55	55	87	64	63	122	82	92
18	36	34	53	55	55	88	64	63	123	82	92
19	36	34	54	55	55	89	64	63	124	82	92
20	38	35	55	55	55	90	64	63	125	82	92
21	38	35	56	55	55	91	64	63	126	82	92
22	39	37	57	57	57	92	66	70	127	89	98
23	39	37	58	57	57	93	66	70	128	89	98
24	40	37	59	57	57	94	66	70	129	89	98
25	40	39	60	57	57	95	66	70	130	89	98
26	41	39	61	57	57	96	66	70	131	89	98
27	41	40	62	57	57	97	66	70	132	89	98
28	41	40	63	57	57	98	66	70	133	89	98
29	42	41	64	59	59	99	67	74	134	98	107
30	42	41	65	59	59	100	67	74	135	98	107
31	42	41	66	59	59	101	67	74	136	98	107
32	44	43	67	59	59	102	67	74	137	98	107
33	44	43	68	59	59	103	67	74	138	98	107
34	44	43	69	59	59	104	67	74	139	98	107
35	45	45	70	59	59	105	67	74	140	98	107

Appendix A (2)

Feeding allowance (g/b/day) of 308 & 708 broiler breeder females from 126- to 160-day-old, pre peak of egg production and lighting programme

Age		Feed allowance (g/b/day)		Lights		Total hours	Pre peak feed		
Week	Day	308	708	on	off		% Production	Feed g/b/day	
								308	708
18	126	103	98	06.30	14.30	8	5	140	137
18.1	127						7	141	137
18.2	128						9	142	139
18.3	129						11	143	141
18.4	130						13	144	143
18.5	131						15	145	144
18.6	132						17	146	145
19	133	109	107				19	147	146
19.1	134						21	148	148
19.2	135						23	148	148
19.3	136						25	149	149
19.4	137						27	150	150
19.5	138						29	151	151
19.6	139						31	152	152
20	140	114	114				33	153	153
20.1	141						35	154	154
20.2	142						37	155	155
20.3	143						39	156	156
20.4	144						41	157	157
20.5	145						43	159	158
20.6	146						45	161	158
21	147	116	116				47	162	159
21.1	148						49	163	160
21.2	149						51	164	160
21.3	150	118	117				53	165	161
21.4	151						55	166	162
21.5	152	122	120				57	166	163
21.6	153						59	167	164
22	154	124	122	04.30	15.30	11	60	168	
22.1	155						61		165
22.2	156						63		166
22.3	157	127	125				65		167
22.4	158						67		168
22.5	159	131	127						
22.6	160	135	129						

Appendix A (3)

Feeding allowance (g/b/day) and lighting programme for 308 & 708 broiler breeder females from 161-day-old to the end of lay

Age		Feed g/b/day		Lights		Total hours
Week	Day	308	708	on	off	
23	161	136	131	04.30	16.30	12
23.1	162					
23.2	163					
23.3	164	137	133			
23.4	165					
23.5	166	138	135			
23.6	167					
24	168	140	137	04.30	17.30	13
24.1	169	See pre peak				
24.2	170					
24.3	171					
24.4	172					
24.5	173					
24.6	174					
25	175			04.30	18.30	14
26	182					

Appendix B

Sample preparation for fat analysis (Rapid Soxhlet extraction)

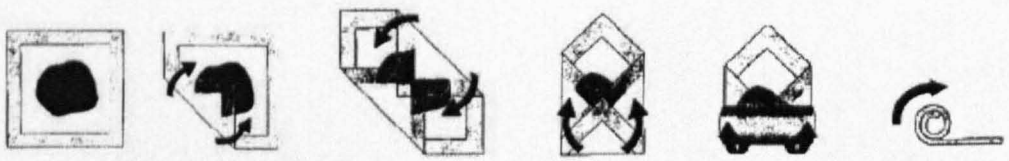
- 2g of ground and oven dried sample was weighed onto a $10cm^2$ of filter paper
- After folding the paper into a loose parcel, it was placed in an extraction thimble and plugged with cotton wool
- 155 ml of petroleum ether was added to an oven-dried, pre-weighed Soxtherm flask
- As the thimble was placed into the wire holder in the top of the flask, the Soxtherm machine was run for fat extraction
- After extraction; with no ether remaining, the flask contains the extracted fat was dried at 103°C for an hour
- After cooling in the desiccator, the flask was re-weighed
- Fat percentage was calculated by:

$$\text{Fat \%} = \frac{\text{Wt of extracted fat} \times 100\%}{\text{Sample Wt}}$$

Appendix C

Sample preparation for fat analysis (Microwave drying and nuclear magnetic resonance; NMR)

- 2 square sample pads were placed on the balance pan in the microwave compartment and tare, then a sample of 3.0±1 g was weighed onto the top sample pad
- Both pads were then removed and placed on pre-refrigerated chopping board and sample was spread around the top sample pad as thinly and evenly as possible
- The bottom sample pad was carefully then removed and placed on top and the two pads were firmly pressed.
- The sample pads and the dried sample were then removed and placed on the centre of one sheet of Trac film placed on a flat surface and then it was rolled as the following



- The rolled sample was the placed in the Trac tube; positioned previously in the holder on the Trac station, and pressed firmly to the bottom of the tube to the mark on the Trac station that shows the maximum height of the pressed sample
- The Trac tube containing the pressed sample then was put into the reading area of the Smart Trac NMR, then the analysis was run
- At the end of the analysis the fat content was displayed on the screen as a percentage.